Biomarker based therapies in high risk cancer patients - MACC1 as molecular target

DISSERTATION

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Zusammenfassung

Trotz intensiver Forschung zeigen die meisten Krebstherapien weiterhin geringen Behandlungserfolg. Keine Ausnahme bildet hier das kolorektale Karzinom: Es ist die zweithäufigste Todesursache aller krebsassoziierten Tode weltweit. Insbesondere, die Ausbildung von Fernmetastasen stellt eine der größten Herausforderung in der Krebstherapie dar. Es fehlen hier weiterhin ausreichend verlässliche und effiziente Biomarker zur Prognose des Krankheitsverlaufes oder zur Auswahl bestimmter Patienten für spezifische Behandlungsstrategien (Prädiktion).

Metastasis-associated in colon cancer 1 (MACC1) ist ein inzwischen etablierter, prognostischer, prädiktiver und kausaler Biomarker für verschiedene Tumorentitäten. Auf molekularer Ebene kann es sowohl verschiedene Zielgene wie z.B. MET, NANOG oder SPON2 induzieren als auch unterschiedliche Signalwege wie MEK/ERK und AKT/β-catenin beeinflussen. Auf diese Weise fördert es Zellproliferation, -migration und -Koloniebildung sowie Tumorprogression und Metastasierung *in vivo*. Diese Arbeit sollte neue Strategien erforschen diese Prozesse durch die Inhibition von MACC1 zu unterbinden.

Wir haben dafür zwei bestimme Screening-Methoden zugrunde gelegt. Zum einen wollten wir neue und stärkere transkriptionelle Inhibitoren für MACC1 identifizieren. Zum anderen wollten wir weitere Ebenen des MACC1 Signalnetzwerks und somit neue therapeutische Interventionspunkte entdecken. Mithilfe des ersten Verfahrens und anschließender Validierung konnten wir nahezu alle klinisch angewendeten Statine als potente transkriptionelle Inhibitoren von MACC1 nachweisen. Mit den beiden stärksten Inhibitoren, Fluvastatin und Atorvastatin, konnten wir zeigen, dass Statine sowohl die Proliferation und Koloniebildung *in vitro* als auch Tumor Wachstum und Metastasierung *in vivo* MACC1-spezifisch reduzieren. Die *in vivo* Versuche wurden mit einer Statindosis äquivalent zur Standardtherapie bei Reduzierung der Blutlipide im Menschen durchgeführt.

Im zweiten Screening konnten wir phosphotyrosin (pY)-abhängige Interaktionen von MACC1 mit essentiellen Signalmolekülen identifizieren: SHP2, GRB2, SHC1, PLCG1 und STAT5B. Interessanterweise, führte Mutation der Bindungsstellen zu einer verringerten Aktivität des MACC1-induzierten ERK Signalwegs sowie reduzierter Zellmigration und -proliferation. Unsere Daten weisen zudem darauf hin, dass MACC1 in Abhängigkeit verschiedener Rezeptor-Tyrosinkinasen (z.B. MET und EGFR) neue SHP2/SRC/ERK und PKA/SRC/CREB Signalkaskaden orchestriert, was zu einem malignen Phänotyp führt. Gezielte Intervention mit Inhibitoren für MET, MEK, SHP2 und SRC konnte dadurch die MACC1-abhängige Koloniebildung von Darmkrebszellen reduzieren. Diese Ergebnisse zeigen somit neue therapeutische Interventionspunkte auf und stellen darüber hinaus eine hervorragende Basis für weitere Untersuchungen dar. Diese sollten sich am besten mit Kombinations-

behandlungen mit weiteren zielgerichteten Inhibitoren (z.B. gegen GRB2 oder PKA), potentiellen MACC1 pY-Antikörpern oder den Statinen befassen.
Die zusätzliche Erforschung der spatiotemporalen Organisation des MACC1 Signalosoms und der assoziierten Signalkaskaden, insbesondere *in vivo*, wird voraussichtlich das volle Potential von MACC1 als therapeutisches Target ausschöpfen können. Wir empfehlen zudem Statine bereits in der Krebstherapie bzw. -prävention, besonders bei MACC1-stratifzierten Patienten, auch als Monotherapie anzuwenden.

Abstract

Despite intensive research, many cancer treatment strategies present unsuccessful. This is reflected in colorectal cancer (CRC) as the second leading cause of cancer associated deaths worldwide. Here, the development of distant metastasis represents a major challenge in therapy. In addition, reliable and efficient biomarkers for early prognosis of disease course or selection of patients for specific treatment (prediction) remain scarce.

Metastasis-associated in colon cancer 1 (MACC1) has been established as prognostic, predictive and causal biomarker for several tumor entities. It has been found to induce different target genes such as MET, NANOG and SPON2 and affect several signaling pathways including MEK/ERK and AKT/β-catenin. Thus, it promotes cell proliferation, migration and colony formation as well as tumor progression and metastasis formation *in vivo*. This study intended to explore new strategies to inhibit these processes by targeting MACC1.

We employed two distinct screening methods to find novel, more potent transcriptional inhibitors of MACC1 and illuminate the MACC1 signaling landscape to uncover new drug intervention points. With the first screening and subsequent validation, nearly all clinically employed statins were revealed as potent MACC1 transcriptional inhibitors. Chosen as strongest inhibitors, Fluvastatin and Atorvastatin showed MACC1-specific reduction of proliferation and colony formation *in vitro* as well as restriction of tumor growth and metastasis formation *in vivo* at doses equivalent to human standard lipid reduction therapy.

Moreover, we identified phosphotyrosine (pY)-dependent interactions of MACC1 with crucial signaling molecules: SHP2, GRB2, SHC1, PLCG1 and STAT5B. Mutation of the interaction sites abrogated MACC1-dependent ERK signaling as well as cell migration and proliferation. Our data further suggest that MACC1 governs SHP2/SRC/ERK and PKA/SRC/CREB axes conferring a malignant phenotype in response to different receptor tyrosine kinases such as MET and EGFR. Targeted intervention with inhibitors of MET, MEK, SHP2 and SRC restricted MACC1-dependent colony formation. These results indicate new drug intervention points for MACC1 signaling and provide an excellent baseline for further investigations of combinatorial treatments with other targeted inhibitors (*e.g.* GRB2, PKA), potential MACC1 pY-antibodies or statins.

Additional research about the spatiotemporal organization of MACC1 signalosome formation and downstream signaling, particularly *in vivo*, will reveal the entire potential of MACC1 as therapeutic target, whereas statins should already be considered for cancer therapy or prevention, especially in patients stratified for MACC1 expression.

1. Introduction

1.1 Cancer hallmarks

Cancer results from a malignant transformation of healthy cells endowing them with specific features widely acknowledged as hallmarks of cancer [1,2]: These cells gain the ability to evade or circumvent the tightly controlled mechanisms of cell growth (proliferation) and death (apoptosis). They develop self-sustaining capabilities including continuous proliferative signaling, avoiding and preventing immune attacks as well as rewiring their metabolic pathways and angiogenesis. Beyond that, they reach a state of immortality and proceed to infiltrate further tissues and organs, processes commonly known as invasion and metastasis. Tumor promoting inflammation as well as genomic instability or mutation represent, both, prerequisite and self-sustaining features in the multistep and multifactorial process of cancer development: pro-inflammatory signals secreted by immune cells (*e.g.* growth factors, proangiogenic factors, etc.) can support the acquisition of hallmark features in chronic inflammations and, further, even promote these in neoplastic lesions. Moreover, cells can coincidentally acquire growth advantage over others by random mutations whereas tumor cells actively fuel this development by disturbing genomic surveillance and maintenance [1,2]. The previously described processes appear in different cellular contexts and organs giving rise to a broad variety of distinct cancer entities: liquid cancers as leukemia and lymphomas or solid cancers of *e.g.* lung, liver and the gastro-intestinal tract [1,3]. The following chapters will shed more light on the cascade of tumor development and metastasis with specific regard to colorectal cancer (CRC).

1.2 Tumorigenesis and progression of CRC

1.2.1 Origin and mutational background

Mutations demonstrate a key event in the origin and development of cancer and colorectal cancer, respectively [1,2,4]. Several factors and mechanisms play a role in the generation of these mutations: defects during cell division (mitosis) such as chromosomal missegregation, recombination between homologous chromosomes or dysfunction in chromosomal protection and damage response mechanisms lead to aberrant chromosome numbers (aneuploidy), gene amplifications and monoallelic (loss of heterozygosity) or biallelic gene deletions, respectively. This chromosomal instability is one of the major contributors to the CRC mutational landscape [5].

Furthermore, inactivation of enzymes involved in DNA repair such as mismatch repair (MMR) adenosinetriphosphatases (ATPases) and base excision repair genes lead to the

accumulation of DNA errors and mutation of the affected gene. MMR deficiency (dMMR) and subsequent mutations occur more frequently in highly repetitive DNA motifs called microsatellites. This is, therefore, termed the microsatellite instability (MSI) phenotype. Hypermethylation of cytosine residues in gene promoters leads to transcriptional inhibition and represents the central mechanism of MMR epigenetic silencing [6,7]. Increasing age, injury or chronic inflammation of the colon can elevate promoter methylation [8,9]. Especially, the methylation of cytosine-guanosine dinucleotides (CpG islands) abundant in several gene promoters occurs in a higher frequency. This CpG island methylator phenotype (CIMP) defines a subset of CRC [7].

The acquired mutations mentioned above, mostly arise through sporadic somatic events: In about 70% of all CRC cases solely somatic events account for the induction of cancer development, whereas in 10-30% somatic mutations in addition to a familial predisposition initiate this process. Only 5-7% of all CRCs represent hereditary diseases: most of them belong to the hereditary nonpolyposis colorectal cancer (HNPCC or Lynch-syndrome) or the familial adenomatous polyposis (FAP) syndrome [10]. Generally, the mutational processes can be facilitated by endogenous or exogenous mutagenic stimuli continuously challenging the DNA, such as reactive oxygen species (ROS), ultraviolet B (UVB) radiation as well as heterocyclic amines from tobacco smoke or processed meat [11-13].

Only mutations affecting critical genes disturb the regulation of cellular growth and death leading to uncontrolled proliferation and spread [1,2]. Among our approximately 20,000 genes, 138 putative driver genes were identified by now: The 64 oncogenes gain their function by mutations rendering them permanently active or overexpressed. Loss-of-function mutations in the tumor suppressor genes (74 genes) diminish or terminate their cellular gatekeeping abilities [3]. Only three mutated driver genes can suffice to initiate tumorigenesis and progression of CRC, but individual CRCs, usually, harbor up to 15 driver gene mutations [3,14-16]. Fearon & Vogelstein firstly described this in a model as a series of sequential mutational events in specific genes [4].

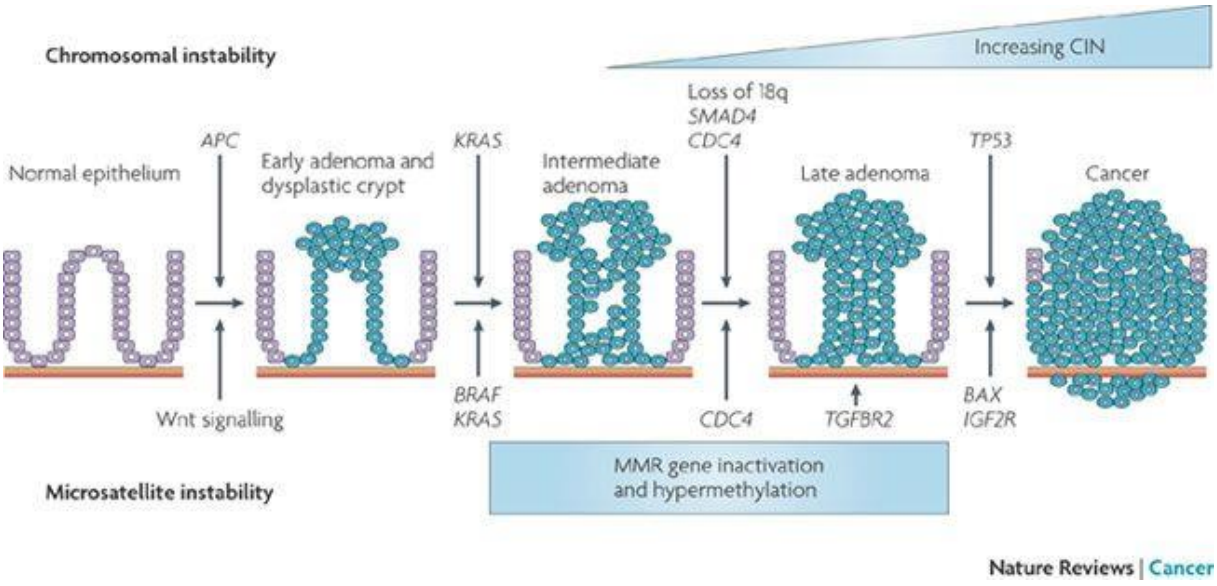


Fig. 1: Adenoma-carcinoma sequence: CRC is a multistep process dependent on the mutational aberration of several crucial genes involved in the control of cell growth. Dysfunctional cell division leads to chromosomal instability (CIN) or epigenetic silencing through methylation of certain gene promoters followed by (in)activation of these genes and an accumulation of mutations. This figure depicts an overview of this process and involved key factors, which is further explained in the text. APC - adenomatous polyposis coli; Wnt – wingless/integrated; KRAS - Kirsten rat sarcoma viral oncogene homolog; BRAF - v-Raf murine sarcoma viral oncogene homolog B; 18q – q-arm of chromosome 18; SMAD4 - SMA & MAD homolog 4; CDC4 - cell division control protein 4; TGFBR2 - transforming growth factor beta receptor 2; TP53 - tumor suppressor P53; BAX - Bcl-2-associated X protein; IGF2R - insulin-like growth factor 2 receptor; MMR - mismatch repair [17].

Widely recognized as CRC initiating step, a functional loss of the adenomatous polyposis coli (APC) gene is found in about 70% of all sporadic cancers whereas the germline mutation characterizes the FAP syndrome. Although most FAP syndrome patients show secondary mutations of the remaining wild-type allele, it is not essential for diminished APC function since it displays an autosomal-dominant phenotype. The APC protein mainly works in a multiprotein complex determining β-catenin for degradation and, consequently, retaining it from its oncogenic functions. The FAP syndrome confers a life-time risk of 80-100% for CRC and patients undergo prophylactic resection of the colon [18]. APC represents a prominent example to highlight the effects genetic mutations inflict on cellular signaling (see further 1.2.2).

Further initial steps in the cancer cascade constitute the inactivation of MMR genes such as MutL homolog 1 (MLH1), MutS protein homolog 2 (MSH2) or MutY homolog (MYH). 15-25% of sporadic CRCs display epigenetic silencing of MMR genes and germline mutations in these characterize the HNPCC [6,7]. Additional mutations in key oncogenes such as Kirsten rat sarcoma viral oncogene homolog (KRAS) or v-Raf murine sarcoma viral oncogene

homolog B (BRAF) as well as tumor suppressors such as tumor suppressor p53 (P53) or phosphatase and tensin homolog (PTEN) further contribute to CRC progression [4,6,17].
The majority (50-60%) of CRCs follow the adenoma-carcinoma sequence whereas the serrated neoplasia pathway and colitis-associated CRC development represent alternative, more recently acknowledged routes of CRC progression. On the one hand, serrated polyps usually endowed with BRAF mutations and increasing CIMP grow into malignant carcinomas [19]. On the other hand, chronic inflammation confers a continuous proliferative, anti-apoptotic and mutagenic environment leading to CRC development under additional tumor suppressor inactivation (*e.g.* P53) or oncogene (*e.g.* KRAS) activation [2,20-22].

1.2.2 Deregulated molecular signaling

All genetic alterations in cancer are reflected in aberrant cellular signaling pathways [23]. Gene mutation and expression profiles alone are insufficient to explain particular phenotypes in the multistep and multilayer process of cancer development [24,25]. In fact, the number of genetic mutations exceeds the number of signaling pathways. Therefore, mutations of different genes in individual signaling cascades usually share the same outcome: deregulation of a determined cellular process such as proliferation or apoptosis. So far, 9 different pathways have emerged as central nodes in cancer signaling. In addition to the multiple layers of each individual pathway, several crossing points allow for inter-pathway regulation and feedback (Fig. 2) [26].

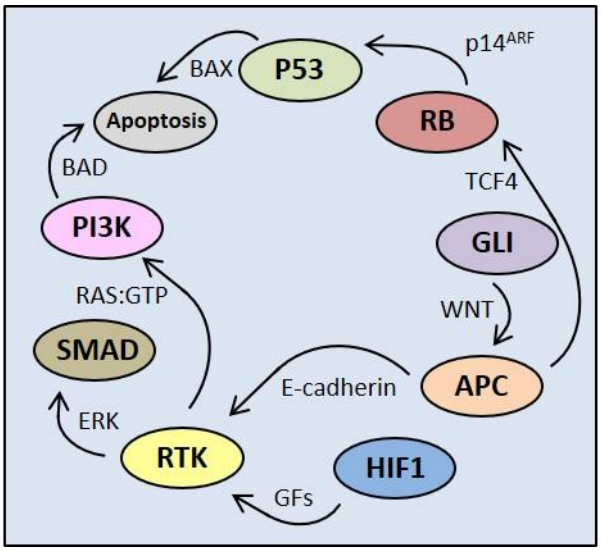


Fig. 2: Central cancer signaling pathways: Schematic representation of 9 crucial pathways (colored boxes) deregulated in cancer development. Genetic alteration of genes involved in these pathways leads to aberrant signaling and subsequent phenotypic changes such as increased cell proliferation or motility. Some of these pathways share common factors enabling significant crosstalk among them. Crossing-points and some of their mediators are indicated with arrows. P53 - tumor suppressor P53, Rb - retinoblastoma protein, $p14^{ARF}$ - ARF tumor suppressor, GLI - glioma-associated oncogene, TCF4 - Transcription factor 4, APC - adenomatous polyposis coli, Wnt - Wingless/Integrated, HIF1 – hypoxia inducible factor 1, GFs – growth factors, RTK – receptor tyrosine kinase, SMAD4 - SMA & MAD homolog, ERK - extracellular signal–regulated kinase, PI3K - phosphatidylinositol 3-kinase, RAS:GTP – active/GTP-bound form of small GTPase RAS, BAD - Bcl-2-associated death promoter (BAD) protein, BAX - Bcl-2-associated X protein. Modified from [26].

The progressing tumor continuously interacts with its microenvironment integrating this information mainly via surface receptors and their downstream signaling [2]. A prominent and important class of receptors are receptor tyrosine kinases (RTKs) comprising different growth factor receptors such as epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR) or MET receptor tyrosine kinase [27]. The proto-oncogene MET encodes a transmembrane tyrosine kinase, which serves as receptor for hepatocyte growth factor/scatter factor (HGF/SF). Therefore, it is also called the hepatocyte growth factor receptor (HGFR) [28,29]. MET activation plays a pivotal role in general epithelial-cell motility as well as growth and survival of epithelial cells and migration of myogenic precursor cells in embryonal development [30-37]. Consequently, overexpression or mutations of MET leading to aberrant, constantly activated signaling profoundly contribute to tumorigenesis [38-42].

Next to a characteristic domain structure with a β-propeller fold accountable for ligand binding, the MET protein contains a distinct binding motif on its intracellular tail: This docking site consists of tyrosine sites Y1349 and Y1356 and surrounding amino acids [43,44]. Phosphorylation of these tyrosine residues upon receptor activation leads to the recruitment of different substrates such as growth-factor-receptor-bound protein 2 (GRB2)-associated

binder 1 (GAB1), GRB2 and phosphatidylinositol 3-kinase (PI3K) with GAB1 as central signal mediator [44-47]. GAB1 provides several tyrosine sites which upon phosphorylation bind further signaling molecules: *e.g.* Src Homology 2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP2), SH2 domain containing transforming protein (SHC1), phospholipase C gamma (PLCG), and signal transducer and activator of transcription (STATs). SHP2, for instance, can activate RAS thereby inducing the extracellular signal–regulated kinase (ERK) pathway, one of the central MET downstream signaling cascades [48-56].

RAS activation is usually catalyzed by the guanine nucleotide exchange factor (GEF) son of sevenless (SOS) [57]. Activated RAS recruits RAF kinases such as BRAF to the plasma membrane leading to their phosphorylation which results in activation. The RAF kinases transduce the activating signals by phosphorylating MAPK/ERK kinases (MEKs) at serine residues in their activation loop (*e.g.* S218 and S222 for MEK1). MEKs can now phosphorylate ERKs (ERK1 – 44 kDa, ERK2 – 42 kDa) at their activating sites threonine (T) 202 and tyrosine (Y) 204. Upon stimulation ERK can translocate to the nucleus and induce several transcription factors such as c-FOS, P53 and c-JUN by phosphorylation. This leads to the expression of target genes crucial for the regulation of proliferation, survival and other important cellular processes. ERK can, additionally, stimulate other downstream effectors such as 90 kDa ribosomal S6 kinases (RSKs) leading to their nuclear translocation and subsequent activation of target genes. Inactivation of the ERK cascade is conferred by different phosphatases such as protein phosphatase 2 (PP2A), receptor-type tyrosine-protein phosphatase R (PTP-SL) and MAPK phosphatases (MKP) or negative feedback via inhibitory phosphorylation of SOS, RAFs or MEKs by ERK [58].

PI3K is another important effector of RTK signal transduction. It consists of a regulatory (p85) and a catalytic (p110α) subunit. Upon recruitment to activated phosphorylation sites of RTKs or adaptor proteins, p85 releases p110α from autoinhibition which can then catalyze the phosphorylation of phospholipids in the plasma membrane generating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [59,60]. PIP3s serve as platform for the binding of further adaptor and signaling molecules such as insulin receptor substrate (IRS), GAB1, PLCγ, 3-phosphoinositide-dependent protein kinase-1 (PDK1) and AKT [61-65]. Stimulation of the central signal mediator AKT via phosphorylation at S473 and T308 leads to its dissociation from the plasma membrane and subsequent activation of several downstream targets in the cytoplasm or nucleus. These target genes are involved in controlling cell proliferation, apoptosis, DNA repair, etc. [59,66-69]. ERK and PI3K signaling share a fair amount of crosstalk on different layers of the pathways resulting in dependencies and redundancies for the control of crucial cellular phenotypes (see Fig. 3) [23,70].

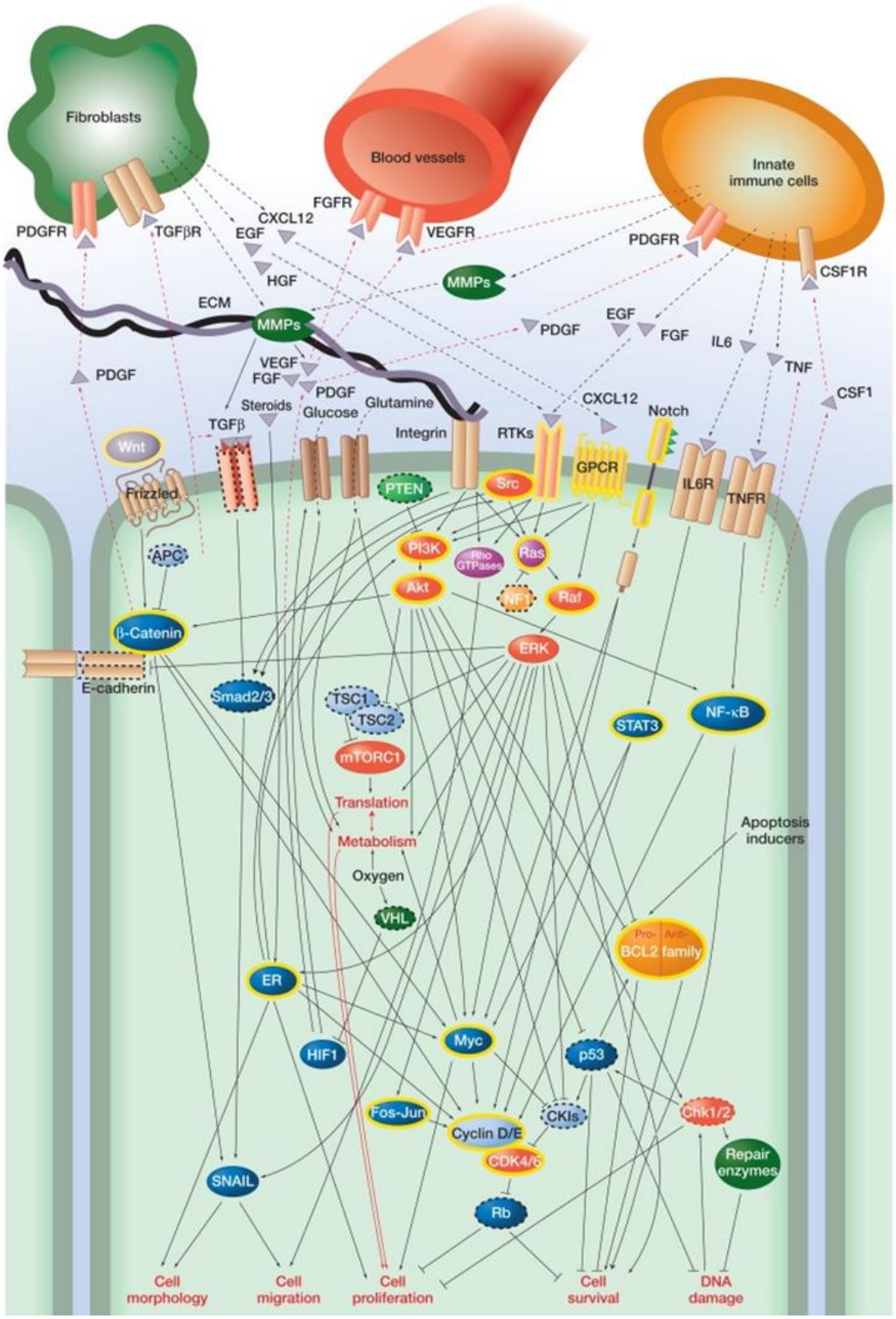


Fig. 3: Overview of crucial signal transduction pathways. Extracellular stimuli are transduced via a variety of signal mediators, mainly transmembrane receptors and respective downstream signaling. This scheme shows the most essential signal transduction pathways with particular respect to ERK and PI3K signaling. It demonstrates the high level of crosstalk and redundancy in cellular signaling. Yellow circles indicate oncogenic proteins while dashed outlines mark tumor suppressors. RTKs – receptor tyrosine kinases; PDGF(R) - platelet-derived growth factor (receptor); TGFβ(R) - transforming growth factor beta (receptor); EGF(R) - epidermal growth factor (receptor); FGF(R) - fibroblast growth factor (receptor); VEGF(R) - vascular endothelial growth factor (receptor); CSF1(R) - colony stimulating factor 1 (receptor); IL6(R) – interleukin

6 (receptor); TNF(R) – tumor necrosis factor (receptor); CXCL12 - C-X-C motif chemokine 12; ECM – extracellular matrix; MMPs – matrix metalloproteases; GPCR - G protein-coupled receptor; PTEN - phosphatase and tensin homolog; P53 - tumor suppressor P53; Rb - retinoblastoma protein; APC - adenomatous polyposis coli; HIF1 – hypoxia inducible factor 1; SMAD2/3 - SMA & MAD homolog 2/3; ERK - extracellular signal–regulated kinase, PI3K - phosphatidylinositol 3-kinase; NF-1 - Neurofibromin 1; RAS - rat sarcoma viral oncogene homolog; RAF - v-Raf murine sarcoma viral oncogene homolog; ERK - extracellular signal–regulated kinase; STAT3 - signal transducer and activator of transcription 3; PI3K - phosphoinositide 3-kinases; AKT – protein kinase B; APC - adenomatous polyposis coli; SRC - non-receptor tyrosine kinase SRC; TSC1/2 - tuberous sclerosis 1/2; mTORC1 - mammalian target of rapamycin complex 1; VHL - von Hippel–Lindau tumor suppressor; BCL2 - B-cell lymphoma 2; NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells; CDK4/6 - cyclin-dependent kinase 4/6; CKIs - cyclin-dependent kinase inhibitors; CHK1/2 - checkpoint kinase 1/2; SNAIL - zinc finger protein SNAI1; ER – estrogen receptor [23].

ERK and PI3K are both able to induce β-catenin signaling via different routes demonstrating the complex hierarchy and substitutability of molecular pathways [71-77]. Wnt/β-catenin signaling plays a crucial role in embryonal development, cell differentiation and stem cell maintenance, which includes the activation of various target genes [78,79]. A destruction complex of β-catenin represents the main hub of downstream signaling. It involves the APC protein and two continuously active serine/threonine kinases (casein kinase 1 alpha/delta (CK1α/δ) and glycogen synthase kinase 3 beta (GSK3β)) scaffolded by Axin. The kinases phosphorylate β-catenin on distinct serine and threonine sites which leads to the recruitment of β-TrCP, part of an E3 ubiquitin ligase complex, determining it for proteasomal degradation [80,81]. Inactivation of the destruction complex through ligand binding to the Wnt receptor frizzled or inhibition (*e.g.* GSK3β) as well as mutation (*e.g.* APC) of single components leads to accumulation and nuclear translocation of β-catenin. Here, it engages T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) and further transcription factors for the expression of target genes involved in many processes such as proliferation, cell cycle and migration [18,78,81-84].

1.2.3 Invasion & Metastasis

During tumorigenesis and carcinoma development, cells acquire additional features enabling them to disseminate from the primary tumor site and spread to distant tissues and organs. This process of metastasis formation was assumed to occur late in the adenoma-carcinoma sequence, but emerging evidence points to the additional possibility of early dissemination [85,86]. The formation of metastatic colonies requires overcoming several barriers during this invasion-metastasis cascade [87-89]: certain genes and genetic alterations equip the affected cells with motility and invasive abilities allowing them to invade surrounding tissue and intravasate to the circulating blood system. Here, further characteristics ensure survival of immune attacks or sheer stress and permit adhesion as well as extravasation to the

distant site where the cells usually reside in a quiescent state of dormancy. Only those cells with the ability to persist, switch back to a proliferative state, and reinitiate tumor growth, will form metastasis. These cancer stem cells (CSCs) display genetic and epigenetic characteristics comparable to common stem cells [86,90].

In addition, secreted factors from the primary tumor can reach distant sites and prepare a suitable microenvironment for disseminating cells: the pre-metastatic niche [91]. The aforementioned aspects support the "seed and soil" hypothesis suggested by Paget already at the end of the 19th century: only certain cancer cells ("seed") show matching compatibility and adaptive programs with the microenvironment of specific target sites ("soil") [87]. This theory partially applies to CRC: even though CRC cells would not show high adaptive potential for liver tissue, the overwhelming number of tumor cells reaching this site via the colon clearing portal vein determine the liver as predominant metastatic site [88]. Further following the blood flow, the lung was found as secondary dissemination site [92]. Over the last years, an increasing number of cases with peritoneal metastasis, cancerous outgrowth in different tissues of the bowel cavity, *e.g.* omentum, mesentery or diaphragm, have been documented. The rising diagnoses are most likely due to improved preoperative imaging techniques and subsequently higher attention for this topic. Recent studies show an approximate incidence of 8% for peritoneal metastasis from CRC [93,94].

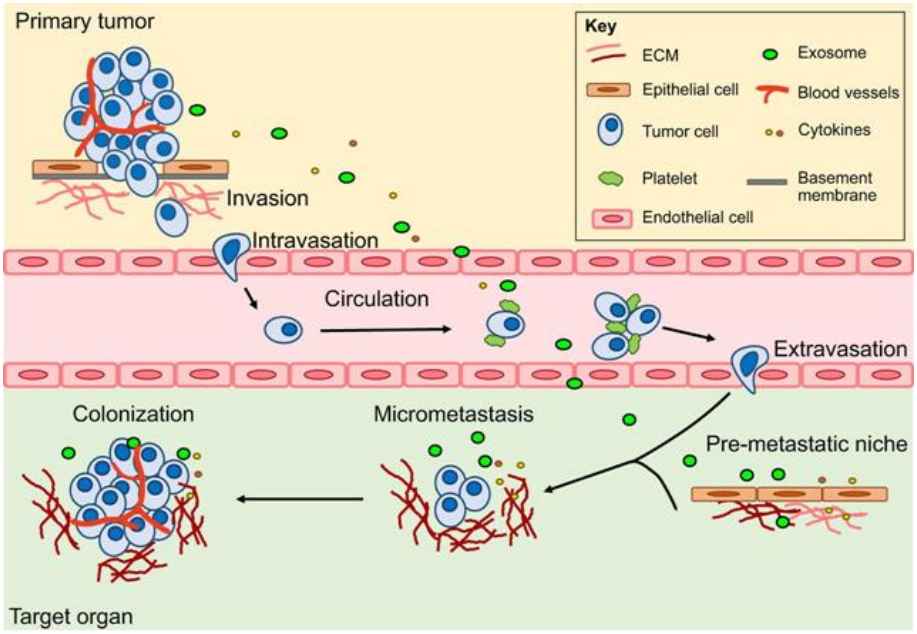


Fig. 4: Invasion-metastasis cascade. Dissemination and colonization of distant tissues involves different molecular mechanisms. Tumor cells need to invade surrounding tissue and further penetrate the circulatory system (intravasation). Here, they have to circumvent sheer stress and immune destruction to adapt and infiltrate the distant site (extravasation). Metastasis can form out of persistent (dormant) cells with growth initiating ability. Secreted factors by the primary tumor can already provide a suitable microenvironment for the disseminated cells at the distant site (pre-metastatic niche). ECM – extracellular matrix [95].

Still, there are several molecular mechanisms determining CRC metastasis: The epithelial-to-mesenchymal transition (EMT) plays a central role in equipping cells with a migratory phenotype [90]. During EMT, cells dissolve their cell-cell contacts, individualize and rearrange their cytoskeleton as well as cellular polarity thereby establishing motility characteristics. These processes can be triggered through different stimuli: *i.a.* transforming growth factor β (TGFβ)-, RTK-, integrin and Wnt signaling regulate EMT-governing transcription factors (TFs) such as zinc finger protein SNAI1 (SNAIL), zinc finger protein SNAI2 (SLUG), Twist basic helix-loop-helix transcription factor 1 (TWIST1) and zinc finger E-box binding homeobox 1 (ZEB) [96].

The EMT program also induces the expression of extracellular matrix degrading proteases such as matrix metalloproteinase (MMP) -2 and -9 [96]. MMPs support the metastatic process by degrading physical barriers and proteolytic activation or inactivation of several essential factors such as TGFβ, vascular endothelial growth factor (VEGF), E-Cadherin or MET [97]. MET is found to be overexpressed or continuously activated in different cancer entities including CRC [40,98]. Downstream signaling of MET induces central mitogenic, anti-apoptotic and pro-migratory pathways such as ERK and PI3K (see 1.2.2) [99]. Moreover, MET is a transcriptional target of the metastasis inducer metastasis associated in colon cancer 1 (MACC1) which will be elucidated in detail later (1.3.2) [100].

Further pacemakers of CRC metastasis belong to the S100 protein family: S100A4, identified as target of β-catenin signaling, can induce migration *in vitro* and liver metastasis *in vivo,* whereas S100A8 contributes to establishing the pre-metastatic niche in the liver [101-103].

Over 50% of patients with CRC develop distant metastasis which drastically affects patient survival (see 1.2.4) [104-108]. This highlights the necessity for new and improved therapy. The following chapters will discuss treatment strategies for CRC and CRC metastasis, respectively.

1.2.4 Current state of therapeutic intervention

The previously described molecular mechanisms exert distinct histopathological patterns. These classify disease stage and thereof treatment strategy. Stage I and II CRC is locally confined thereby partially invading the colon tissue layers, whereas stage III and IV include the invasion to surrounding tissues and lymph nodes (III) as well as metastasis to distant sites (IV) [109]. Tumor stage at the time of diagnosis strongly determines overall survival [110,111]: The 5-year survival rate for patients with early stage CRC (stage I) is around 85%, but decreases drastically with tumor progression. It declines to 50% for patients with progressed CRC which show tumor invasion to the lymph nodes at time of diagnosis (stage

III) and drops to approximately 10% with the development of distant metastasis (stage IV) [104,112].

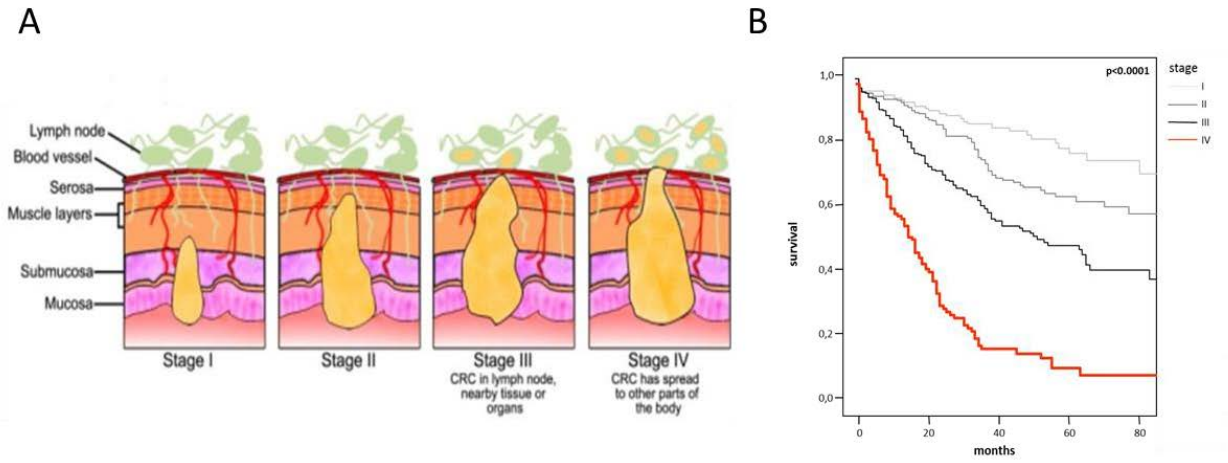


Fig. 5: Tumor staging and associated survival. The multistep process of CRC progression is reflected in a histopathological classification associated with patient survival rates. **A)** In the early stages the tumor invades locally through mucosa, submucosa and muscularis propria (stage I) to the outermost layers of the colon (stage II) whereas the following stages show invasion of local lymph nodes (stage III) and metastasis to distant sites (stage IV) [113]. **B)** Patients in stage I and II demonstrate an average 5-year survival of approximately 85% and 70%, respectively. This drastically drops with stage progression to about 50% in stage III and below 10% in stage IV. CRC - colorectal cancer [104].

First line of therapy represents resection of tumor and metastases. In stage IV multidisciplinary discussion is warranted to evaluate resection of metastasis. Here, systemic chemotherapy can reduce the metastatic burden to alleviate metastasis resection [114,115]. Stage III and high risk stage II (perforation of outermost layers of colon or rectum, lymphovascular invasion (LVI), perineural invasion (PNI)) patients can also benefit from adjuvant chemotherapy [116,117]. Present treatment regimens comprise combinations of cytotoxic (irinotecan, oxaliplatin) and cytostatic (capecitabin/5-fluoruracil (5-FU), trifluridin) drugs and additional supporting agents (leucovorin, tipiracil): FOLFOX regimen (leucovorin, 5-FU and oxaliplatin), the FOLFIRI regimen (leucovorin, 5-FU and irinotecan), and the XELOX regimen (oxaliplatin and capecitabine) [118-123]. Some cases, predominantly rectal cancer, require neoadjuvant (previous) chemo-/radiotherapy to decrease tumor or metastasis size rendering them manageable for resection [124,125].

During stage progression, tumor cells acquire an increasing amount of mutations. Across several tumor entities, individual tumors can harbor up to 15 different driver gene mutations, but also a lot of additional “passenger mutations” shaping (epi)genetically unique cancers: Most entities, including CRC (microsatellite stable, MSS), show between 25 and 75 non-

synonymous mutations per tumor, whereas lung cancer or melanoma display even higher numbers around 150 mutations per tumor, reflecting the strong influence of potent mutagens (UVB and tobacco smoke, respectively) in these entities. Consequently, CRC with MSI exhibit an extraordinarily high mutation frequency between 500 and 1500 mutations per tumor. Considering that each single tumor develops its individual mutation pattern, these facts demonstrate the huge genetic heterogeneity between different tumor entities, but especially, within each entity itself (intertumoral heterogeneity) [3,16,126].

Moreover, the widely acknowledged and established concept of intratumoral heterogeneity reflects the clonal divergence within each individual tumor: cancer cells divide and accumulate further mutations thereby continuously differentiating from the originating cell [127-130]. Here, CSCs, primarily identified by their tumor initiating abilities, might represent a common denominator and determinator of intratumoral heterogeneity: their self-renewing potential and production of differentiated progeny leads to high subclonal diversity [131,132]. Furthermore, CSCs display insensitivity to chemotherapy as well as targeted approaches, probably fueled by their increased expression of antiapoptotic and multidrug transporter proteins. Consequently, tumor heterogeneity and, specifically, CSCs contribute to cancer therapy resistance [132-138]. Accumulating evidence indicates dynamic flexibility of tumor heterogeneity: subclonal diversity can increase under chemotherapeutic pressure and tumor cells might undergo reversible states of drug-tolerance [139,140].

Considering the huge heterogeneity across CRCs, the adverse side effects of systemic chemotherapy and increasingly detailed knowledge about cancer signaling pathways, molecular targeted therapy has emerged over the last years [141-145]. Currently, different antibodies are employed subsequent or in addition to conventional chemotherapy which inhibit downstream function of growth factors or growth factor receptors: bevacizumab (VEGF antibody), cetuximab or panitumumab (EGFR antibodies) show benefits for previously treated patients [119-122,146-149]. Further, aflibercept (a decoy receptor for VEGF-A, VEGF-B and PGF (placental growth factor)) and ramucirumab (an antibody against VEGFR-2) are combined with chemotherapy [150,151]. Regorafenib (a multikinase inhibitor) is used in patients displaying or developing treatment resistance [152].

Additional kinase or receptor kinase inhibitors, such as trametinib (against MEK), dabrafenib (against BRAF) and trastuzumab or lapatinib (against human epidermal growth factor receptor 2, HER2) show promising potential in combinatorial treatment based on different therapy resistance mechanisms [153-156]. The recently emerging immune checkpoint inhibitors, such as pembrolizumab, provide new possibilities for therapy, particularly for CRC with MSI. MSI tumors show – probably facilitated through their high mutational load – a high infiltration and adaption to the immune system: The microenvironment of MSI tumors

presents an unusually high expression of immune checkpoint ligands, including programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) which explains a stronger susceptibility to checkpoint inhibitors [157].

More inhibitors remain under preclinical or clinical evaluation: nintedanib and famitinib target angiogenesis via fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), VEGF, FMS-like tyrosine kinase 3 (FLT-3) and rearranged during transfection receptor tyrosine kinase (RET) signaling [158,159]. The alkylating drug temozolomide and the monoclonal antibody MABp1 against interleukin 1α might become interesting in the nearer future [160,161].

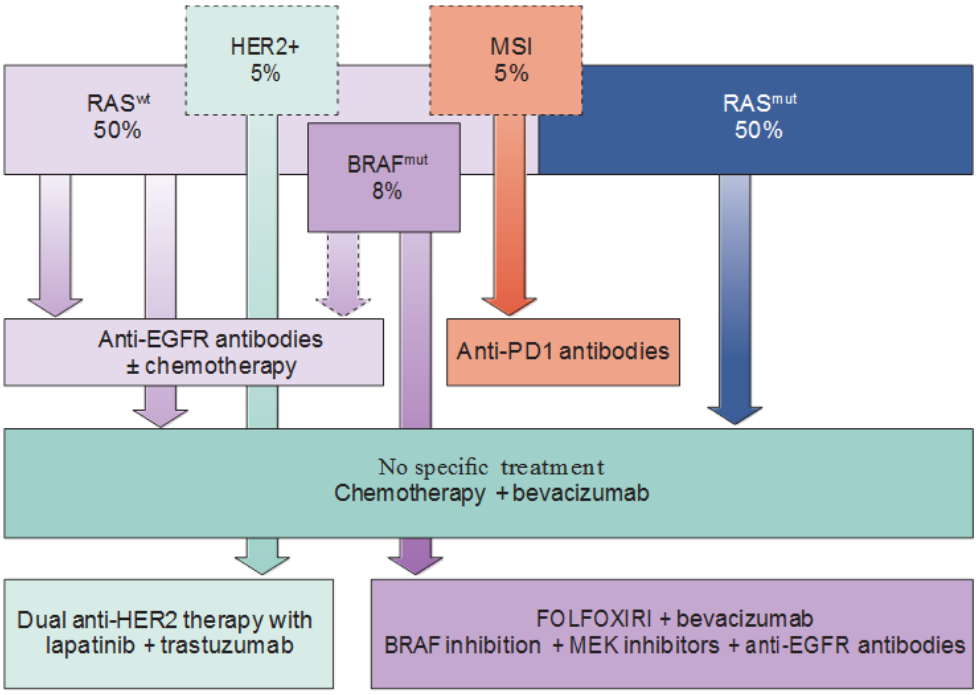


Fig. 6: CRC treatment strategies. The usually employed treatment regimens for CRC (FOLFOX, FOLFIRI, XELOX) can be adjusted with respect to currently established, predictive biomarkers. Patients with RAS mutation will not respond to EGFR antibody therapy, but RAS wild-type patients with either MSI, BRAF mutation or HER2 overexpression might benefit from selective inhibitor treatment (*e.g.* PD-1 antibody for MSI). RAS^{wt} - wild-type rat sarcoma viral oncogene homolog; RAS^{mut} - mutated rat sarcoma viral oncogene homolog; HER2+ - human epidermal growth factor receptor 2 positive; MSI - microsatellite instability; $BRAF^{mut}$ - mutated v-Raf murine sarcoma viral oncogene homolog B; EGFR - EGFR - epidermal growth factor receptor; PD1 - programmed cell death protein 1; MEK - MAPK/ERK kinases; FOLFOXIRI - folinic acid + fluorouracil + oxaliplatin + irinotecan [162].

Despite all the efforts, cancer cells demonstrate therapy resistance due to their highly sophisticated and complex signaling networks. On the one hand, there is a high level of redundancy between signaling pathways controlling proliferation and survival as well as crosstalk among them. On the other hand, cellular signaling is rewired upon therapeutic

intervention: lacking feedback inhibition can reactivate targeted pathways or stress response pathways are induced [26,163-165]. Alternatively, extracellular stimuli from the tumor microenvironment can trigger cellular responses circumventing the inhibited pathway. Clonal divergence might have already led to cell clones in the tumor with growth signals independent of the targeted pathway or expressing drug-resistant target proteins. Cancer cells also might adapt to treatment by accidental or epigenetic activation of crucial pathways [166-168].

CRC remains the second leading cause of cancer-associated deaths worldwide. It accounts for approximately 10% (~900,000 deaths/year) of all cancer-related deaths by a yearly incidence of about 1.8 million people [169]. Especially, the development of distant metastasis represents a major challenge in CRC therapy: the 5-year survival of about 10% could not be significantly improved over the last decades [106-108,170].

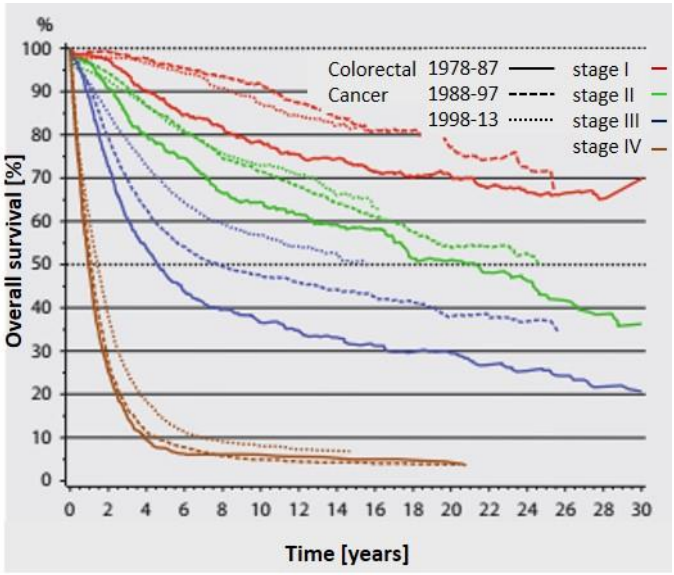


Fig. 7: Survival of CRC patients over time and disease stage. While overall survival could be strongly increased for CRC patients of stage I-III (red, green, blue) over the last 30 to 40 years, stage IV (brown) patients still show very low survival rates [170].

Many therapeutic agents show only partial effectiveness while exerting tremendous side effects due to their cytotoxic properties. Molecular targeted therapy only affects a subset of patients. More importantly, both treatment strategies struggle with therapy resistance [143,144,166-168]. In addition, reliable and efficient biomarkers for early prognosis of disease course or selection of patients for specific treatment (prediction) remain scarce. The most commonly employed prognostic and/or predictive biomarkers are MMR status (MSI vs MSS) and mutations of RAS as well as BRAF [121,171-175]. For instance, stage II CRC patients with MSI have a favorable prognosis compared to MSS patients and present no

benefits from 5-FU treatment [171,176,177]. Newer revelations indicate prognostic or predictive potential for expression of HER2, epiregulin (EREG) or amphiregulin (AREG) as well as different microRNAs (miRNAs) [178-182]. Recently, combining of different biomarkers and molecular features resulted in consensus molecular subtypes (CMS), which should enhance patient stratification and targeted therapy [183]. This approach underlines the necessity for the establishment and application of new biomarkers for cancer treatment.

These biomarkers optimally fulfil all of the following criteria: 1) it is a driver of CRC and CRC metastasis (causal biomarker); 2) it identifies the population at high risk for patient stratification and potential prevention of the imminent disease (prognostic biomarker); 3) it determines therapy response (predictive biomarker) to specify clinical research and design personalized treatment strategies; and 4) it could serve as promising drug target [3,162].

1.3 A novel prognostic, predictive and causal biomarker: MACC1

1.3.1 Prognosis & Prediction

In 2009, our group analyzed tissue samples of normal colon mucosa, primary tumors and their metastases. Interestingly, the primary tumor tissue of non-metastasized patients which developed metastases later on showed an overexpression of an unknown gene. This newly identified gene was named metastasis-associated in colon cancer 1 (MACC1). The analyses revealed a significantly higher expression of MACC1 in malignant tissue compared to normal tissue. Most importantly, metachronously metastasizing tumors showed remarkably higher levels of MACC1 messenger RNA (mRNA) in the primary tumors than those without the development of metachronous and synchronous metastases. High MACC1 mRNA levels are also associated with significantly reduced metastasis-free survival, and the 5-year overall survival dramatically differs between patients with low and high MACC1 levels in the primary tumor: 80% and 15%, respectively [100]. Several follow-up studies corroborated our findings of the prognostic characteristics of MACC1 for metastasis and patient survival in more than 20 solid tumor entities [90,112,184-206]. Also, MACC1 transcript or protein levels in liquid biopsies (*e.g.* patient blood) can be employed for prognosis of tumor progression, metastasis formation and patient survival in different solid tumor entities such as pancreatic, gastric, lung and breast cancer [207-212]. In addition, high MACC1 expression is able to predict *e.g.* disease recurrence after resection of lung adenocarcinoma, CRC liver metastases or hepatocellular carcinoma (HCC) dependent liver transplantation [186,213,214].

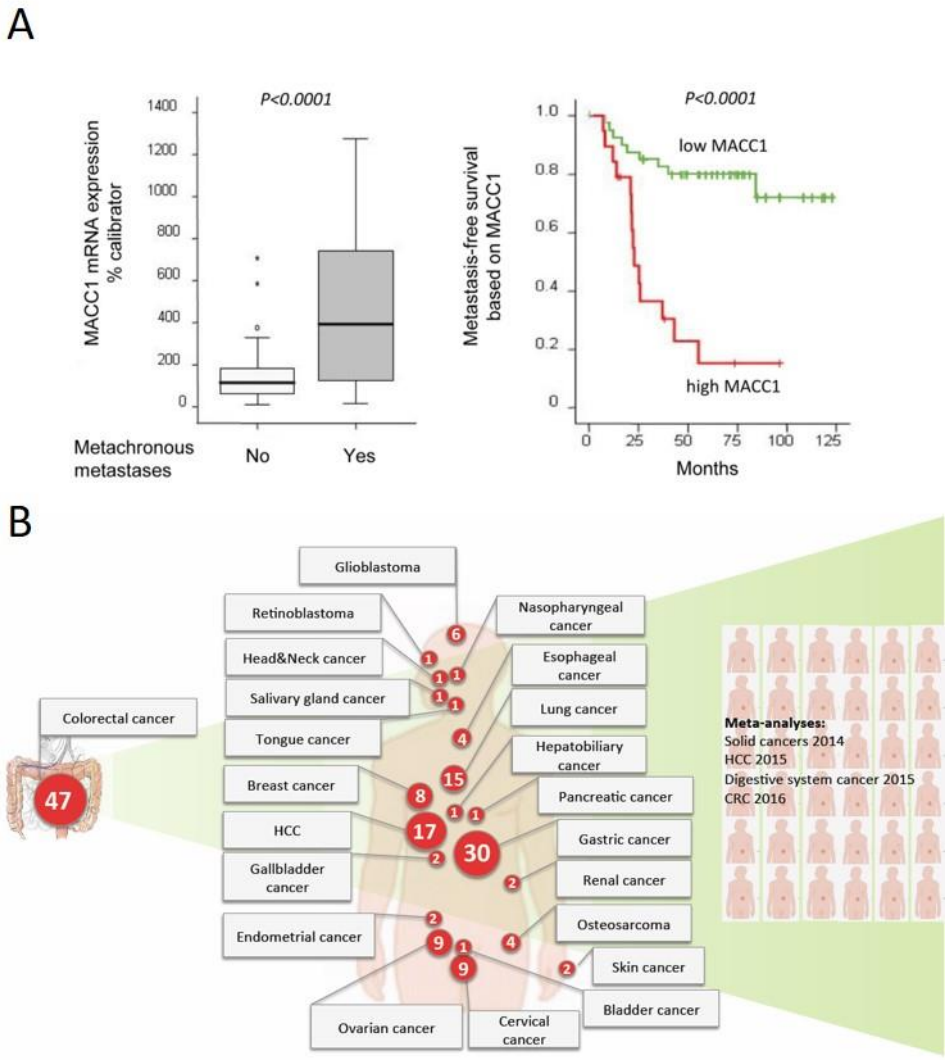


Fig. 8: The prognostic value of MACC1. A) The analysis of primary tumor tissues displayed a higher MACC1 expression in metastasizing tumors compared to those without metachronous metastases. This difference in MACC1 expression could also predict the metastasis-free survival of the respective patients [100]. **B)** This prognostic value of MACC1 was confirmed in CRC and over 20 further tumor entities. HCC - hepatocellular carcinoma; CRC – colorectal cancer; MACC1 – metastasis associated in colon cancer 1 [215].

Furthermore, MACC1 serves as predictive biomarker for different therapeutic drugs (cisplatin, oxaliplatin, 5-FU, gemcitabine) in several entities such as tongue squamous cell cancer, glioblastoma multiforme (GBM), ovarian cancer, gastric cancer, lung cancer, pancreatic cancer and CRC [208,216-224]. It further predicts treatment response for temozolomide/Endothelial-Monocyte-Activating Polypeptide–II (EMAP-II) in GBM [225]. Moreover, high MACC1 expression was predictive for poor outcome after neoadjuvant

chemo-radiotherapy for rectal cancer and after cryoablation therapy for advanced HCC [226,227].

The role of MACC1 as prognostic and predictive biomarker already indicates its critical function in tumor progression and metastasis. Beyond that, MACC1 expression was found to increase during adenoma to carcinoma progression and particularly accumulate at the invasive front in tumor buds or in circulating tumor cells (CTCs), disseminated by the primary tumor. These features combined with its motility inducing function designate MACC1 as causal driver of invasion and metastasis [100,212,228-232].

1.3.2 Signaling landscape and functional impact of MACC1

MACC1 promotes and participates in several cancer hallmark processes: Most importantly, MACC1 induces proliferation, migration, invasion, and colony formation in several solid cancer entities and promotes tumor growth and liver metastasis in different mouse models [100,208,221,225,233-242]. MACC1 is a transcriptional activator of MET, thereby triggering downstream MEK/ERK and PI3K/AKT signaling [99,100].

Overexpression of MACC1 led to an upregulation of MET and resulted in increased cell motility, invasion, wound healing and metastasis. Reciprocally, the knock down of MACC1 reduced MET expression, cell motility and proliferation. MACC1 activates MET expression by binding to its promoter, which was abrogated by mutating the Src homology 3 (SH3) domain or PxxP motif on MACC1. Additionally, the MACC1-hyperactivated ERK pathway, in turn, increased MACC1 levels resulting in a potential positive feedback loop [100,191,243,244].

MACC1 governed signaling promotes EMT and stem cell characteristics which represent crucial features of cancer progression and dissemination (see 1.2.3) underlining its important role in these processes [195,223,235,236,245,246]. As discussed above (1.2.3), certain transcription factors control the expression of crucial genes for EMT. In fact, TWIST1/2 expression and downstream targets were shown to depend on MACC1. ZEB2-inhibited EMT, including target gene expression, was rescued by MACC1 [247-249]. MACC1 further regulates the expression of several genes involved in EMT such as E-Cadherin, N-Cadherin, and MMPs, mainly through AKT signaling [228,235,237,250-252]. Concomitantly, MACC1-activated AKT can inhibit GSK3β thereby stabilizing β-catenin and leading to increased expression of target genes such as MYC, Cyclin D1/E and MMPs [237,250].

The MACC1/AKT/β-catenin axis also confers chemoresistance and stemness characteristics such as sphere formation by activating EMT [219,221,223,225,253]. Moreover, MACC1 directly enhances stemness by regulating the pluripotency markers NANOG and octamer-binding transcription factor 4 (OCT4) [239]. The expression of additional stem cell genes

shows correlations with MACC1 in several tumor entities, characterized by higher invasive potential and poor prognosis [254-257].

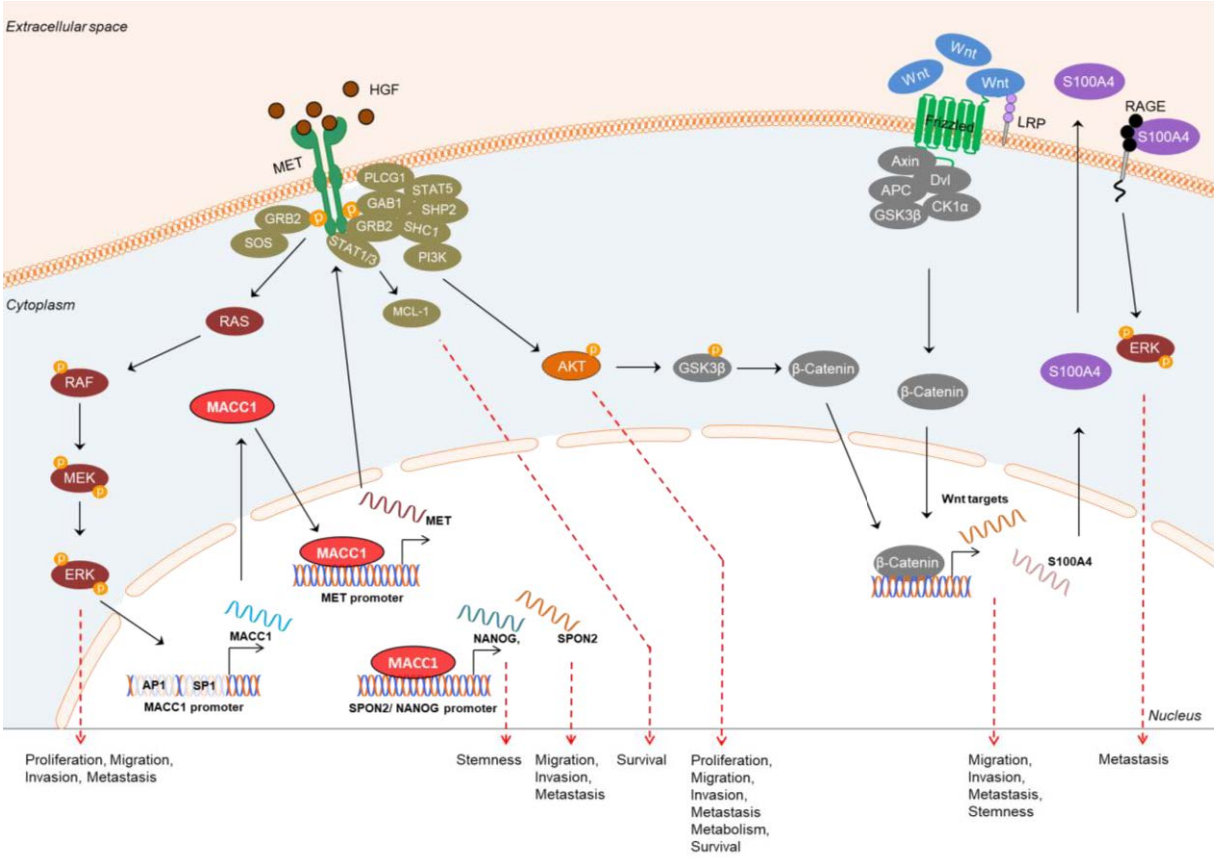


Fig. 9: The molecular functions of MACC1. MACC1 acts as driver of tumor progression and metastasis through different mechanisms. It induces the expression of crucial target genes such as MET, NANOG and SPON2, thereby ultimately promoting proliferation, migration, invasion, stemness and metastasis. In this context, MACC1 influences several pathways including MEK/ERK, AKT/β-catenin and STAT/MCL-1. MACC1 – metastasis associated in colon cancer 1; MET - MET receptor tyrosine kinase; HGF – hepatocyte growth factor; GRB2 - growth factor receptor-bound protein 2; SOS - son of sevenless; RAS - rat sarcoma viral oncogene homolog; RAF - v-Raf murine sarcoma viral oncogene homolog; MEK - MAPK/ERK kinases; ERK - extracellular signal–regulated kinase; AP1 - activator protein 1; SP1 - specificity protein 1; STAT1/3/5 - signal transducer and activator of transcription 1/3/5; GAB1 - GRB2-associated-binding protein 1; SHC1 - SHC-transforming protein 1; SHP2 - SH2 domain-containing phosphatase 2; PI3K - phosphoinositide 3-kinases; MCL-1 - induced myeloid leukemia cell differentiation protein; NANOG - homeobox protein NANOG; SPON2 - spondin 2; AKT – protein kinase B; GSK3β - glycogen synthase kinase 3 beta; Dvl - dishevelled protein; APC - adenomatous polyposis coli; CK1α - casein kinase I alpha; LRP - lipoprotein receptor-related protein; RAGE - receptor for advanced glycation endproducts; S100A4 - S100 calcium-binding protein A4.

MACC1 contributes to metabolic rewiring of tumor cells mainly by enhancing the Warburg effect associated with augmented tumor progression, poor prognosis or increased resistance to apoptosis as well as drug treatment [242,258-261]. It supports tumor growth by generation and reorganization of blood and lymphatic vessels inducing angiogenesis, vascular mimicry

and lymphogenesis through the elevated expression of several involved key factors such as TWIST1 and VEGF A/C/D [245,247,248,256,262].

Chronic inflammation can establish a malignant microenvironment fostering neoplasia and cancer development [2]. Recently, MACC1 was associated with inflammatory bowel disease associated dysplasia, a risk factor for CRC, and might contribute to a tumor promoting environment through macrophage recruitment via TWIST1/2 activation [189,247,263-266]. Otherwise, MACC1 confers protective features to cancer cells, thereby avoiding apoptosis or immune destruction. Through activation of different pathways (PI3K/AKT, MEK/ERK and STAT), it modulates the expression or activity of several proteins involved in apoptosis or immune protection such as FAS ligand (FASL), tumor necrosis factor related apoptosis inducing ligand (TRAIL), induced myeloid leukemia cell differentiation protein (MCL-1), BAX/BAD, BCL2 and caspase 8/9/3/7 as well as PARP [208,233,241,250,267-270]. Besides apoptosis, MACC1 is able to control further tightly monitored mechanisms such as the cell cycle: Mainly through regulation of PTEN/AKT with subsequent expression of crucial target genes (cyclin B, D1, D2, E, MYC and SPON2), MACC1 governs G_0/S-phase transition and cell cycle progression thereby initiating cell growth [221,236,237,241,250,251,267,271-273].

The previous discussion demonstrates the pivotal role of MACC1 in the initiation and advancement of several cancer hallmark features, including different signaling mechanisms. This highlights the enormous potential for targeted intervention of MACC1 and downstream signaling, which will be further discussed in the following chapter.

1.3.3 Structural features and inhibitory potential of MACC1

The MACC1 cDNA consists of 2559 base pairs which encode for an 852 amino acid protein with a molecular weight of 97 kDa. In the genome it is localized on locus 7p21.1. This region (7p21) harbors further genes involved in tumorigenesis or metastasis such as TWIST1 or integrin beta 8 (ITGB8) [191]. The MACC1 promoter (-992 until -18 bp upstream of the transcription start site, TSS) drives transcription under the control of transcription factors AP-1, SP1 and C/EBP which individually contribute to MACC1 expression [238]. Based on these findings, our group aimed to identify transcriptional inhibitors of MACC1. By high-throughput drug screening (HTS) we could determine Lovastatin and Rottlerin as effectors of MACC1 promoter activity and subsequent expression. Both inhibitors could restrict MACC1-dependent cell migration *in vitro* as well as metastasis formation in xenografted mice [274]. Additionally reported expression regulation via the transcription factor YB1 (in lung cancer) or intracellular calcium signaling (in gastric cancer) indicates tissue dependent specificities

[246,275]. Further investigations should lead to a better understanding of the spatiotemporal expression of MACC1.

On the posttranscriptional level, the long non-coding RNA (lncRNA) MACC1-AS1 leads to increased MACC1 expression through MACC1 mRNA stabilization [259]. Contrary, zinc finger protein 36 homolog (ZFP36), an RNA destabilizing protein, and many different miRNAs, such as miR-200a, miR-218 or miR-338-3p, negatively regulate MACC1 expression and concomitant functions [234,249,262,276-289]. Moreover, successful RNA interference (RNAi) of MACC1 expression and associated functions via small interfering RNA (siRNA) or small hairpin RNA (shRNA) *in vitro* an *in vivo* further reinforces the promising role of MACC1 as valuable target for cancer therapy [100,219,222,225,233,237,240,250].

The translated MACC1 protein expresses a distinct structural architecture with five predicted domains: At the N-terminus two domains resemble known structures of zonula occludens 1 and uncoordinated protein 5 (ZU5), or of uncoordinated protein 5 (Unc5), p53-induced death domain protein 1 and ankyrins (UPA) [146]. Further to the C-terminal side, MACC1 harbors a SH3 domain as well as a tandem of death domains (DD) [100,290,291]. All these domains demonstrate functional abilities involved in protein-protein interaction or protein recruitment [292-295]. In this regard, MACC1 contains more protein interaction motifs, such as the clathrin box, NPF and DPF tripeptides as well as proline-rich sequences (PxPxP, KxxPxxP) and several serine (Ser, S), threonine (Thr, T) or tyrosine (Tyr, Y) sites accessible for phosphorylation [100].

Fig. 10: MACC1 domain architecture. MACC1 contains several distinct, predicted domains characterizing it as potential interactor in different processes. The ZU5, UPA, SH3 and death domains are known to play important roles in in protein-protein interaction or protein recruitment. Further structural motifs such as the Clathrin box, NPF and DPF tripeptides, proline-rich sequences or several potential phosphorylation sites (S, T, Y), additionally, indicate its role as signaling and adapter molecule. The most crucial proline-rich sequence and previously identified, important Y-sites (Y673, Y695, Y793) are depicted in this figure. ZU5 - zonula occludens 1 and uncoordinated protein 5 resembling domain; uncoordinated protein 5 (Unc5), p53-induced death domain protein 1 and ankyrins resembling domain; SH3 - Src homology 3 domain.

Especially, tyrosine phosphorylation represents a crucial mechanistic switch in signal transduction. Phosphorylation of specific Y-sites mostly leads to an activation of the respective protein. Phosphorylated tyrosines (pY), additionally, serve as docking sites for SH2 domain containing proteins, thereby fostering downstream signaling [296-299]. Mutation of the SH3 domain and the proline rich motif (PxxP) or distinct tyrosine sites interfered with

MACC1 function *in vitro* and *in vivo,* further indicating the encouraging potential of MACC1 as therapeutic target in cancer and cancer metastasis, respectively [100,300].

1.4 Aims of the thesis

Over the last years MACC1 has emerged as prominent prognostic marker for metastasis formation and metastasis free survival in many different solid tumor entities. Moreover, its role in prediction of therapy response has become evident. Most importantly, MACC1 has been proven to be a driver of tumor progression and metastasis thereby representing a promising target for targeted intervention. The reduction of MACC1 levels via RNAi or transcriptional inhibition could restrict tumor growth and metastasis formation *in vivo*. So far, only two MACC1 transcriptional inhibitors have been identified highlighting the necessity for additional, possibly more potent treatment strategies. Unfortunately, the revelation of the precise MACC1 protein structure remains elusive which could facilitate the generation of potential MACC1 protein inhibitors. Additionally, the MACC1 phospho-interactome as well as signaling pathways associated with MACC1 functionality have to be further elucidated. This current background determines the main objectives of this study as follows:

- Identification of novel MACC1 transcriptional inhibitors from the European Molecular Biology Laboratory (EMBL) library to reveal additional, possibly more potent inhibitors. This included the evaluation of these inhibitors *in vitro* for the reduction of MACC1 expression and MACC1 associated functions as well as *in vivo* validation for their ability to restrict MACC1-induced tumor progression and metastasis formation in a xenografted mouse model.
- Identification and characterization of tyrosine phosphorylated MACC1 protein-protein interactions and associated molecular pathways. This comprised the validation and functional investigation of identified bindings including their respective interaction sites and, additionally, illumination of the MACC1 involvement in cellular signaling cascades to reveal potential intervention points for targeted treatment.

2. Material & Methods

2.1 Cell lines and growth conditions

All human cancer cell lines used in this study were either purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) or German Collection of Microorganisms and Cell culture (Leibnitz Institute DSMZ, Braunschweig, Germany) (summarized in table 1). They were cultivated in DMEM or RPMI1640 medium (Thermo Fisher Scientific, Waltham, Massachusetts) supplemented with 10% fetal bovine serum (FBS; Bio & Sell, Feucht, Germany). Plastic consumables from TPP (Trasadingen, Switzerland), BD Biosciences (Heidelberg, Germany) or Greiner BioOne (Kremsmünster, Austria) were used for cell maintenance and experiments. Cells were kept at 37°C in a humidified incubator with 5% CO_2. The MycoAlert Mycoplasma detection kit (Lonza, Basel, Switzerland) was regularly used to test the cell lines for mycoplasma.

Table 1: Summary of used human cancer cell lines

Cell line	Medium	Characteristics
CRC cell lines:		
SW480	RPMI + 10% FBS	adherent, colon, colorectal adenocarcinoma
SW620	DMEM + 10% FBS	adherent, colon, colorectal adenocarcinoma, derived from metastatic site: lymph node
HCT116	RPMI + 10% FBS	adherent, colon, colorectal carcinoma
SW48	RPMI + 10% FBS	adherent, colon, colorectal adenocarcinoma
HT-29	DMEM + 10% FBS	adherent, colon, colorectal adenocarcinoma
Gastric and pancreatic cancer cell lines:		
MKN45	DMEM + 10% FBS	adherent, stomach, gastric adenocarcinoma
BxPC3	RPMI + 10% FBS	adherent, pancreas, pancreas adenocarcinoma

2.2 Virus production and generation of stably modified cell lines

For transfection, 1.5×10^7 HEK cells were seeded. After 24 h, cells were transfected using 2.85 ml serum-free medium, mixed with 90 µg of polyethylenimine and kept at room temperature for 5 min. 30 µg of lentiviral plasmids with packing vectors (20 µg psPax2, 10 µg pMD2.G) were mixed and incubated at room temperature for 20 min and added to the respective plates. After 48 h of incubation, the supernatant was collected and filtered (0.45 µm filter). The filtered supernatant was loaded on a 20% sucrose cushion and centrifuged at 4°C for 4 h at 28,000 rpm. The viral particles were dissolved in 500 µl sterile PBS and stored at -80 °C. Cells were transduced in 6-well plates with a multiplicity of infection (MOI) less than 10 for each respective well. After 24 h of incubation, virus-containing medium was replaced with the regular medium and the GFP expressing cells were sorted using FACS.

The plasmid RC224774L2 (Origene, Rockville, Maryland) was employed to create cytomegalovirus (CMV) promoter-driven overexpression of MACC1-GFP and GFP control cells (HCT116/MACC1-GFP and HCT116/GFP). Tyrosine mutant cell lines were generated by site-directed mutagenesis (SDM) of the same plasmid using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent, Santa Clara, California) following manufacturer´s instructions. The employed primers are indicated in table 2. HCT116 cells were transfected with the plasmid pGL4.17 (Promega, Fitchburg, Wisconsin) containing the luciferase reporter gene under control of the human MACC1 promoter sequence (-992 to -18 bp upstream of the TSS) to generate HCT116/MACC1p-Luc cells [238]. A CMV promoter-driven luciferase reporter expressing HCT116 cell line (HCT116/CMVp-Luc) was created as described previously [301]. HCT116/MACC1 was generated with the tetracycline inducible vector pCW-GW-rtTA/MACC1-P2A-nLuc for the inducible overexpression of MACC1 with a nanoLuciferase tag separated by a P2A self-cleaving peptide. MACC1 expression, here, is controlled by a tetracycline responsive promoter, PTight, consisting of seven tetracycline operator sequences just upstream of the minimal CMV promoter (lacking the CMV enhancer). The vector was produced by gateway cloning with the entry vector for MACC1 (Y2776; GeneCopoeia Inc., Rockville, Maryland) and the destination vector pCW-GW-rtTA-P2A-nLuc (kindly provided by Dr. Nikolas Gunkel, DKFZ, Heidelberg) using the Gateway™ LR Clonase™ II Enzyme Mix (Invitrogen, Carlsbad, California) according to manufacturer´s instructions. Cells were lentivirally transduced and selected with Blasticidin.

HCT116/MACC1 -/- were generated according to the protocol by Ran *et al.* with the vector pSpCas9(BB)-2A-GFP (Addgene, Watertown, Massachusetts) and the single guide RNAs: sgMACC1 fwd – GTT TGA AGA GTA CCC GGG TTT GG and sgMACC1 rev – ACA TGC CTT GCT CCG TAT GCA GG (Biotez, Berlin, Germany) [302].

SW480/ev and SW480/MACC1 cells with stable overexpression of MACC1 including a V5-Histidin tag were obtained by transfection of pcDNA3.1-V5-His and pcDNA3.1-MACC1-V5-His (Thermo Fisher Scientific, Waltham, Massachusetts) in SW480 cells. Neomycin treatment selected positive clones. Stable overexpression was monitored via regular detection of MACC1 mRNA levels by quantitative real-time polymerase chain reaction (qRT-PCR; see 2.3).

2.3 RNA extraction and qRT-PCR

qRT-PCR was employed to investigate mRNA or DNA expression of different genes. To isolate total RNA, we used the Universal RNA Purification Kit (Roboklon, Berlin, Germany) according to manufacturer´s instructions. RNA samples were eluted with nuclease-free water and quantified using a NanoDrop 1000 Spectrophotometer (Peqlab, Erlangen, Germany). 50 ng RNA were applied to reverse transcription (RT) with random hexamers in a reaction mix (5 mM $MgCl_2$, 1x RT-buffer, 4 mM pooled dNTPs, 1 U/µl RNAse inhibitor and 2.5 U/µL Moloney Murine Leukemia Virus reverse transcriptase; Thermo Fisher Scientific) at 23°C for 15 min, 42°C for 45 min and 99°C for 5 min with subsequent cooling at 4°C for 5 min.
Complementary DNA (cDNA) was amplified in a quantitative polymerase chain reaction (qPCR) employing the LightCycler® 480 (Roche Diagnostics, Risch, Switzerland) with GoTaq® dye (Promega, Fitchburg, Wisconsin) chemistry under following conditions: 95°C for 2 min followed by 45 cycles of 95°C for 7 s, 60°C for 10 s and 72°C for 5 s. Primers were synthesized and HPLC purified by BioTeZ Berlin Buch GmbH (see table 2). Data was analyzed with the LightCycler® 480 Software release 1.5.0SP3 (Roche Diagnostics, Risch, Switzerland). Unspecific amplification products or primer dimers could be identified by the melting curve measured with a continuous temperature increase from 65°C to 95°C with a rate of 0.1°C/s. Duplicate qRT-PCR reaction values were averaged and each mean value of the expressed gene was normalized to the respective controls (*e.g.* solvent). Glucose-6-phosphat-dehydrogenase (G6PD) and ribosomal protein L32 (RPL32) were used as housekeeping genes to calculate relative gene expression.

Table 2: List of employed primers for qRT-PCR and SDM

Gene	Primer	Sequence 5´- 3´
qRT-PCR-primer		
MACC1	MACC1 fwd	TTC TTT TGA TTC CTC CGG TGA
	MACC1 rev	ACT CTG ATG GGC ATG TGC TG
G6PDH	G6PDH fwd	ATC GAC CAC TAC CTG GGC AA
	G6PDH rev	TTC TGC ATC ACG TCC CGG A
RPL32	RPL32 fwd	TAA GCG TAA CTG GCG GAA AC
	RPL32 rev	TAA CCA ATG TTG GGC ATC AA
SDM-primer		
Y379F	Y379F fwd	GGAATTTATGGACCCAAA**TTT**ATCCATCCCAGTTTTACTG
Y789F	Y789F fwd	ATGATGTGGAAACCTGCC**TTT**GATTTTCTGTATACCTGGA

2.4 Protein extraction and standard & digital Western blotting (WB)

WB was performed to analyze protein expression. For protein extraction cells were scraped off in ice-cold RIPA buffer (50 mM Tris, 150 mM NaCl and 1% NP-40, pH 7.5 supplemented with complete protease inhibitor tablets; Roche Diagnostics, Risch, Switzerland, and phosphatase inhibitor tablets; Roche Diagnostics, Risch, Switzerland), lysed for 30 min on ice with intermediate vortexing, and centrifuged for 45 min at maximum speed. After determination of protein concentration using Bicinchoninic Acid Protein Assays Reagent (Thermo Fisher Scientific, Waltham, Massachusetts) according to manufacturer´s instructions, denatured proteins were applied to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and, subsequently, standard WB or digital WB (DigiWest). For standard WB 30 µg of protein were separated by SDS-PAGE with 10% Tris-HCl gels in Tris/glycine/SDS buffer and blotted to PVDF membranes (Bio-Rad Laboratories Inc., Hercules, California) at 2.5 A, and 25 V for 7 min employing the TransBlot® Turbo™ system (Bio-Rad Laboratories Inc., Hercules, California). Membranes were blocked for 1 h at room temperature with 5% bovine serum albumin (BSA) in TBST buffer (10 mM Tris-HCl, 0.1% Tween20 and 150 mM NaCl, pH 7.5). Incubation of membranes with respective primary antibodies (table 3) in 5% BSA TBST solution at 4°C overnight was followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (table 3) in TBST for 1 h at room temperature. Antibody-protein complexes were visualized with WesternBright

ECL HRP substrate (Advansta, Menlo Park, California) and subsequent exposure to CL-Xposure Films (Thermo Fisher Scientific, Waltham, Massachusetts). WB for β-actin and vinculin served as protein loading control. Spot densitometry was performed using ImageJ (version 1.51j8, National Institutes of Health, USA). Signal intensities were normalized to loading controls. Additionally, intensities measured for phosphorylated proteins were normalized to those of total proteins. For reprobing, membranes were stripped according to the mild stripping protocol provided by abcam® (Cambridge, United Kingdom), blocked and again incubated with respective primary and secondary antibodies [303].

For the DigiWest, performed in cooperation with NMI TT Pharmaservies (Reutlingen, Germany), 10 µg of protein were separated by SDS-PAGE with NuPAGE® Novex® 4-12% Bis-Tris protein gels and blotted to PVDF membranes (Merck-Millipore, Burlington, Massachusetts) at 30 V for 75 min. Before cutting each lane in 96 fractions (0.5 mm x 6 mm), proteins were biotinylated on the membrane at room temperature for 1 h in the dark. The proteins of each individual strip are eluted in 96-well plates followed by loading on color-coded streptavidin-coated Luminex® beads (NMI TT Pharmaservices, Reutlingen, Germany). The beads of four initial WB lanes are pooled and aliquoted again in 96-well plates using 384 distinct bead populations. Each bead pool is then subjected to antibody-based immunoassay (1 Ab/well). For this, the bead aliquots (200 beads/well) were washed with PBS + 0.1% Tween20 (PBS-T) incubated with diluted antibody in assay buffer (Blocking Reagent for ELISA; Roche, Basel, Switzerland) supplemented with 0.2% milk powder, 0.05% Tween-20 and 0.02% sodium azide) at 15°C overnight with 750 rpm on a Thermomixer comfort (Eppendorf, Hamburg, Germany). Most important antibodies used in the assay are listed in table 3. Species-specific phycoerythrin (PE)-labelled secondary antibodies (Jackson Dianova, Hamburg, Germany) were added after two PBS-T washing steps and incubated for 1 h at 23°C with 750 rpm. This was followed by two additional washing steps with subsequent read-out in a Luminex® FlexMAP 3D reader (Luminex, Austin, Texas). The respective signals are integrated for quantification. Protein size was determined referring to a set of proteins of known molecular weight. Data was normalized to total protein loaded (streptavidin - phycoerythrin measurement) [304].

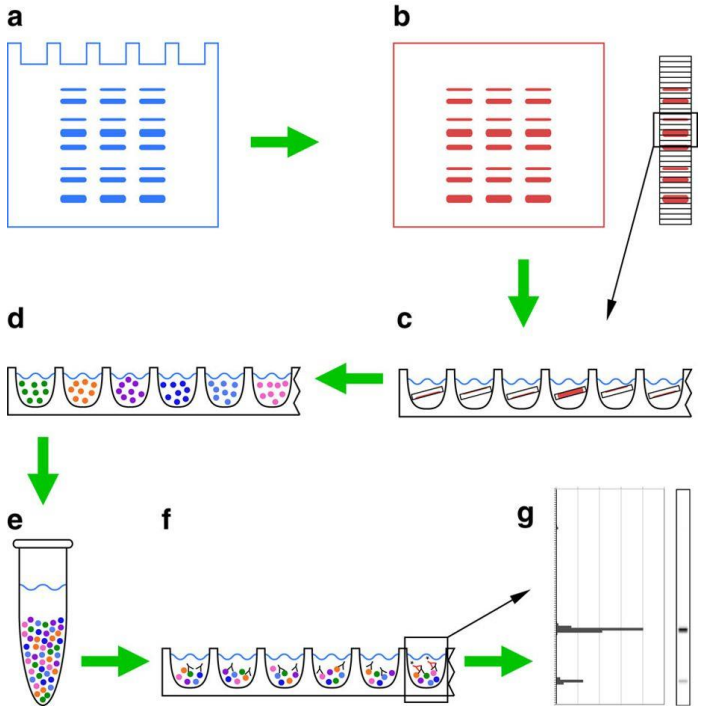


Fig. 11: Digital Western blot principle. Standard Western blot procedure is followed by on-membrane-biotinylation of the proteins. Membranes are then cut in 96 fractions (0.5 mm x 6 mm), distributed to 96-well plates where the proteins are stripped and coupled to color-coded neutravidin-coated Luminex® beads. The beads are pooled and redistributed to 96-well plates to incubate them with primary and secondary antbiodies in an immunoassay and subsequent read-out in a Luminex® reader to evaluate protein expression [304].

Table 3: List of antibodies used in standard and digital Western blotting

Analyte	dilution	antibody	product number/company
Standard WB			
MACC1	1 : 10,000	rabbit, polyclonal	HPA020081 / Sigma-Aldrich
ERK	1 : 1,000	rabbit, polyclonal	#9102 / Cell Signaling
pERK (T202/Y204)	1 : 1,000	rabbit, polyclonal	#9101 / Cell Signaling
MET	1 : 1,000	rabbit, monoclonal	#8198 / Cell Signaling
pMET (Y1234/1235)	1 : 1,000	rabbit, monoclonal	#3077 / Cell Signaling
CREB	1 : 1,000	rabbit, monoclonal	#9197 / Cell Signaling
pCREB (S133)	1 : 1,000	rabbit, monoclonal	#9198 / Cell Signaling
SRC	1 : 2,000	rabbit, monoclonal	#2109 / Cell Signaling
pSRC (Y416)	1 : 1,000	rabbit, polyclonal	#2101 / Cell Signaling
β-actin	1 : 25,000	mouse, monoclonal	A1978 / Sigma-Aldrich
vinculin	1 : 2,000	mouse, monoclonal	V9131 / Sigma-Aldrich

PLCG1	1 : 2,000	rabbit, polyclonal	sc-81 / Santa Cruz Biotech
STAT5B	1 : 1,000	mouse, monoclonal	sc-1656 / Santa Cruz Biotech
SHP2	1 : 1,000	rabbit, polyclonal	sc-280 / Santa Cruz Biotech
SHC	1 : 1,000	rabbit, polyclonal	sc-1695 / Santa Cruz Biotech
GRB2	1 : 1,000	rabbit, polyclonal	sc-255 / Santa Cruz Biotech
Anti-rabbit-HRP	1 : 20,000	goat, polyclonal	W4011 / Promega
Anti-mouse-HRP	1 : 20,000	goat, polyclonal	# 31430 / Thermo Fisher
DigiWest			
β-catenin	1 : 200	rabbit, polyclonal	06-734 / Merck-Millipore
pβ-catenin (S552)	1 : 200	rabbit, polyclonal	#9566 / Cell Signaling
BRAF	1 : 200	rabbit, polyclonal	07-583 / Merck-Millipore
pBRAF (S445)	1 : 100	rabbit, polyclonal	#2696 / Cell Signaling
CREB	1 : 200	rabbit, monoclonal	#9197 / Cell Signaling
pCREB (S133)	1 : 1,000	rabbit, monoclonal	#9198 / Cell Signaling
Cyclin A	1 : 1,000	rabbit, polyclonal	ab53054 / abcam
Cyclin B1	1 : 100	mouse, monoclonal	#4135 / Cell Signaling
Cyclin D1	1 : 200	mouse, monoclonal	#2926 / Cell Signaling
ERK1/2	1 : 200	rabbit, monoclonal	#4695 / Cell Signaling
pERK1/2 (T202/Y204)	1 : 2,000	rabbit, monoclonal	#4370 / Cell Signaling
FAK	1 : 200	rabbit, monoclonal	2146-1 / Epitomics
pFAK (Y397)	1 : 200	rabbit, monoclonal	ab81298 / abcam
pFAK (Y576/Y577)	1 : 200	rabbit, monoclonal	2183-1 / Epitomics
pFAK (Y925)	1 : 200	rabbit, polyclonal	ab38512 / abcam
GSK3β	1 : 200	rabbit, monoclonal	#9315 / Cell Signaling
pGSK3β (S9)	1 : 200	rabbit, polyclonal	#9336 / Cell Signaling
MACC1	1 : 200	rabbit, polyclonal	HPA020081 / Sigma-Aldrich
MEK1	1 : 200	rabbit, polyclonal	#9124 / Cell Signaling
pMEK1/2 (S217/S221)	1 : 200	rabbit, monoclonal	#9154 / Cell Signaling
RSK1	1 : 200	rabbit, polyclonal	06-668 / Merck-Millipore
pRSK1 (T573)	1 : 200	rabbit, monoclonal	ab62324 / abcam
SRC	1 : 200	rabbit, monoclonal	#2109 / Cell Signaling
pSRC (Y416)	1 : 200	rabbit, monoclonal	#6943 / Cell Signaling
TCF4	1 : 200	rabbit, monoclonal	#2565 / Cell Signaling
VASP	1 : 200	rabbit, monoclonal	#3132 / Cell Signaling
pVASP (S157)	1 : 200	rabbit, polyclonal	#3111 / Cell Signaling

2.5 Co-Immunoprecipitation (Co-IP)

To identify or confirm molecular interactions of proteins, Co-IP experiments were conducted. We seeded $5x10^6$ cells per 10 cm dish and let the cells accommodate for 24 h. Cells were washed once with 1x PBS and scratched off in 500 µl ice-cold IP-lysis buffer (20 mM Tris-HCl, pH = 7.5, 150 mM NaCl, 0.1% NP40, 1 mM EDTA, 1% Triton X supplemented with complete protease inhibitor cocktail and phosphatase inhbitors; Roche Diagnostics, Risch, Switzerland). Cell lysis for 30 min with intermediate vortexing was followed by centrifuging for 45 min at 14,000 rpm and 4°C. Supernatants were collected and divided in 2 mg protein containing samples. Proteins were pulled-down with 2 µg of the respective target antibody overnight at 4°C on a rotational shaker. The protein-antibody complexes were incubated and bound with 20 µl of G-agarose beads (Alpha Diagnostic International Inc., San Antonio, Texas) for 4 h at 4°C on a rotational shaker. After five washing steps with 200 µl lysis buffer at 2500 rpm for 5 min at 4°C, we eluted the protein-complexes with 30 µl of LDS-buffer (Thermo Fisher Scientific, Waltham, Massachusetts) including DTT (1:10; AppliChem GmbH, Darmstadt, Germany) at 99°C for 10 min. Following another spin at 2500 rpm for 5 min at 4°C, 20 µl of supernatant were applied to SDS-PAGE and Western blotting (2.4).

2.6 Proliferation and cell viability assay

Cell viability and proliferation was assessed using the IncuCyte® ZOOM System (Essen BioScience, Ann Arbor, Michigan). $4x10^3$ cells/well were seeded in 96-well plates and allowed to accommodate for 24 h. After respective treatments, plates were placed into the IncuCyte® for 72 h – 144 h at 37°C in a humidified incubator with 5% CO_2. The IncuCyte® ZOOM automatically recorded four pictures of each well every two hours which allowed a detailed analysis of the area covered by proliferating cells over time. For data analysis and export the IncuCyte ZOOM software 2016B was used.

2.7 Clonogenic assay

The clonogenic assay was performed to investigate the reproductive viability. In principle, every individual cell is challenged for its ability to divide limitlessly which is judged by their colony forming potential [305-307]. HCT116/GFP and HCT116/MACC1-GFP were seeded in 6-well dishes (400 cells/well) or 24-well dishes (200 cells/well) and allowed to attach for 24 h. After drug treatment, plates were placed into a humidified incubator at 37°C with 5% CO_2 for 7 days. For analysis, medium was removed and the colonies were fixed and stained with a solution of PBS containing 1% Formaldehyde and 0.1% crystal violet. The colony covered area was determined with the colony area plug in and colony number with the particle

analyzing function of ImageJ (version 1.51j8, National Institutes of Health, USA). Results are expressed as area percent normalized to solvent-treated controls.

2.8 Migration assay

The Boyden-chamber assay was performed for evaluation of the migratory potential of cells. Here, the ability of cells to migrate along a chemotactic gradient through the pores of a trans-well membrane was assessed. Previous to seeding, cells were serum starved overnight. Afterwards, $2.5x10^5$ cells (24-well format) or $5x10^4$ cells (96-well format) were seeded in 2% FBS containing medium onto the membrane of a trans-well insert. This was placed in a well containing 10% FBS medium. Plates were incubated in a humidified incubator at 37°C with 5% CO_2 for 24 h. For analysis, cells of the bottom well and the bottom side of the membrane were trypsinized and centrifuged for 5 min at 5,000 rpm. The amount of living cell content was determined using CellTiter-Glo® solution (Promega, Madison, Wisconsin) and subsequent luminescence measurement with the Infinite Pro multiplate reader (TECAN, Männedorf, Switzerland).

2.9 Assessment of growth factor signaling

For illumination of the influence of MACC1 on growth factor mediated downstream signaling, we seeded cells in 6-well dishes at a density of $3x10^5$ cells/well. Cells could accommodate for 24 h in a humidified incubator at 37°C with 5% CO_2. After serum starvation for 18 h, cells were treated with 20 ng/ml of respective growth factors (HGF; kindly provided by Prof. Dr. Walter Birchmeier and EGF; Sigma-Aldrich, St. Louis, Missouri) for 0, 1, 8 and 90 min. The treatment was terminated by removing the media and application of ice-cold PBS. Cells were immediately scraped off in ice cold RIPA buffer and then further processed according to WB procedure (2.4).

2.10 High-throughput drug screening (HTS) and *in vitro* validation

For identification of MACC1 transcriptional inhibitors, an HTS was conducted in collaboration with the European Molecular Biology Laboratory [308]. Here, HCT116-MACC1p-Luc CRC cells were seeded in 384-well plates (Perkin Elmer, Waltham, Massachusetts) at 4,000 cells/well. The 4241 compounds were previously distributed on these plates in two different concentrations: 5 µg/ml and 0.5 µg/ml of Prestwick library compounds, and 1 µM and 0.1 µM of NIH and Microsource library compounds, respectively. Incubation of cells with compounds for 24 h at 37°C in a humidified incubator with 5% CO_2 was followed by the assessment of the luminescent signal. For this, plates were treated with 25 µl luminescent reagent for 5 min (BriteLite plus; Perkin Elmer, Waltham, Massachusetts) and measured in an Envision Reader with ultrasensitive luminescence detector (Perkin Elmer, Waltham,

Massachusetts). The most promising compounds were chosen based on the largest ratio between luminescence reduction and cytotoxicity for 72 h assessed by ATP measurement with ATPlite solution (Perkin Elmer, Waltham, Massachusetts).

Statins were purchased from Selleckchem (Houston, Texas) (Atorvastatin, Pravastatin and Pitavastatin) or Thermo Fisher Scientific (Waltham, Massachusetts) (Fluvastatin, Simvastatin, Rosuvastatin, Lovastatin) and stored at -20°C. The 20 mM stock solutions were prepared fresh for every application in dimethylsulfoxide (DMSO). Control cells were always treated with equal amounts of solvent to rule out adverse effects caused by DMSO.

2.11 PamChip® assay

In cooperation with PamGene International B.V. (BJ 's-Hertogenbosch, Netherlands) we conducted the PamChip® kinase microarray assay to evaluate the influence of MACC1 on kinase activity in CRC cells. The array chip contains 144 unique peptide sequences each for Y and S/T kinases immobilized onto a porous membrane. These 15-amino acid sequences correspond to a putative endogenous phosphorylation site and serve as phosphorylation substrate for kinases in the cell lysate (see [309,310]). In presence of ATP and fluorescein-labeled anti-phospho-Y (PY20) or anti-phospho-S/T antibodies plus fluorescein-tagged secondary antibody (STK antibody mix), the phosphorylation was measured in the PamStation®12.

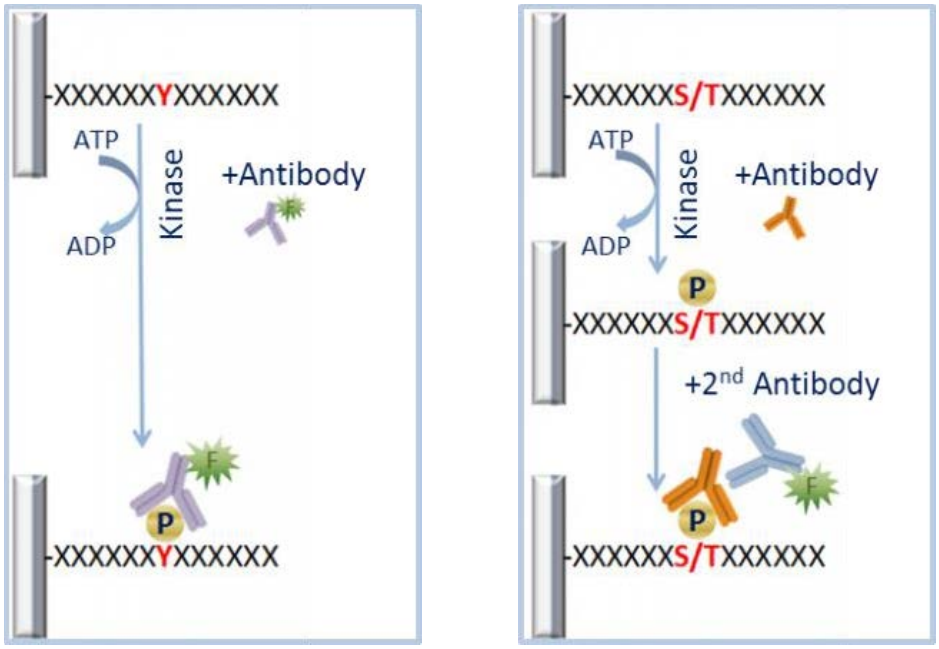


Fig. 11: PamChip® assay principle. Immobilized peptides are phosphorylated by active kinases in whole cell lysates. Phosphorylation is detected by anti-phosphotyrosine or –serine/threonine antibodies with fluorescein-tag or fluorescein-tagged secondary antibody. Measurement is processed in a PamStation®12 [311,312].

For this, we generated lysates of untreated CRC cells modulated in MACC1 expression (SW480/ev & SW480/MACC1, HCT116/GFP & HCT116/MACC1-GFP) with Mammalian Protein Extraction Reagent (M-PERTM; Thermo Fisher Scientific, Waltham, Massachusetts) containing phosphatase and protease inhibitors (Thermo Fisher Scientific, Waltham, Massachusetts). A reaction mix with 5 µg (Y kinase assay) / 0.5 µg (S/T kinase assay) of protein lysate, NEBuffer™ for protein kinases (New England Biolabs, Ipswich, Massachusetts), ATP (400 µM; Sigma-Aldrich, St. Louis, Missouri), 20 µg/mL PY20 (Exalpha Biologicals, Inc., Shirley, Massachusetts) or STK antibody mix (PamGene, BJ 's-Hertogenbosch, Netherlands) was applied to pre-blocked (2% BSA in PBS) array chips. The chips were incubated for 60 min using the PamStation®12 pumping the reaction mixture through the membranes every minute. This was followed by washing and fluorescence measurement.

2.12 Mass spectrometry (MS) screen

For the identification of interactors of tyrosine-phosphorylated MACC1, we conducted a mass spectrometry (MS) screen in collaboration with the lab of Prof. Bernhard Küster (Technical University Munich). Here, we used bead-coupled peptides representing all putative phosphotyrosine (pY) sites of MACC1 as affinity tools to pull down potential binding partners from whole cell lysates.

For this, synthesized peptides (Integrated DNA Technologies, Coralville, Iowa) were linked to NHS-Sepharose beads (Amersham Biosciences, Little Chalfont, United Kingdom) as described previously [313]. Sequences of the most important peptides are summarized in table 6. SW480 and SW620 cells were lysed in IP buffer containing 0.8% NP40, 50 mM Tris-HCl pH 7.5, 5% glycerol, 1.5 mM $MgCl_2$, 150 mM NaCl, 1 mM Na_3VO_4, 25 mM NaF, 1 mM DTT supplemented with protease inhibitors (Sigma-Aldrich, St. Louis, Missouri) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, Missouri). Adjusted cell lysates (5 mg/ml) were incubated with the bead-coupled peptides for 30 min at 4°C with subsequent centrifugation. After washing with IP buffer with successively reduced amounts of NP40 (0.4% and 0.2%, respectively), pulled-down proteins were eluted with LDS sample buffer (Invitrogen, Carlsbad, California) containing 50 mM DTT for 30 min at 50°C. Eluates were alkylated with chloroacetamide (55 mM) and applied to a short SDS-PAGE (approximately 1 cm) in a 4-12% NuPAGE gel (Invitrogen, Carlsbad, California). Here, the proteins were desalted and concentrated for following in gel digestion with trypsin.

Derived peptides were analyzed using an Eksigent nanoLC-Ultra 1D+ (Eksigent, Dublin, California) coupled to an LTQ-Orbitrap XL ETD (Thermo Fisher Scientific, Waltham, Massachusetts). For this, peptides were trapped in a column (100 µm × 2 cm) with self-

packed Reprosil-Pur C_{18}-AQ 5 µm resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) and 0.1% formic acid in HPLC grade water at a flow rate of 5 µL/min. Separation was achieved on an analytical column (75 µm × 40 cm, self-packed with Reprosil-Pur C_{18}-AQ, 3 µm resin; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) with a 110 min gradient of a buffer containing 0.1% formic acid and 5% DMSO in water differing in acetonitrile concentration (gradient: 4-32%) at 300 nL/min flow rate. MS spectra were yielded at a resolution of 30,000 (m/z 400) after accumulation to a target value of 1,000,000.
MaxQuant (version 1.5.3.30.) software was used for peptide identification and quantification. Here, MS/MS data is compared to all canonical protein sequences as annotated in the Swissprot reference database [314,315]. Analysis criteria comprised trypsin as proteolytic enzyme, two missed cleavage sites allowed variance and precursor and fragment ion tolerances of 10 parts per million (ppm) and 20 ppm, respectively. Further, the minimum recognized peptide length was set to seven amino acids as well as the peptide false discovery rate to 1% including common contaminants and reverse identifications.

Table 4: List of crucial MACC1 pY-peptides used in the MS screen

pY-site	Aminod Acid Sequence 5´- 3´
Y365	ATIWD(p)YIHKTT
Y379	IYGPK(p)YIHPSF
Y789	MWKPA(p)YDFLYT

2.13 *In vivo* validation of transcriptional inhibitors

To investigate the effect of identified MACC1 transcriptional inhibitors, drugs were applied to xenografted SCID-beige mice (Charles River, Wilmington, Massachusetts) and tumor growth as well as metastasis formation was measured. Animal experiments were conducted according to the United Kingdom Coordinating Committee of Cancer Research (UKCCCR) guidelines and in cooperation with Experimentelle Pharmakologie & Onkologie Berlin-Buch GmbH (EPO, Berlin, Germany). The State Office of Health and Social Affairs (Landesamt für Gesundheit und Soziales, LaGeSo, Berlin, Germany) granted the animal experiments under the permit Reg0010/19.

2.13.1 Intrasplenal tumor transplantation

$3x10^5$ of HCT116/CMVp-Luc cells were intrasplenically transplanted into 6-week-old female SCID-beige mice (Charles River, Wilmington, Massachusetts) with a 27-gauge needle. For

this, 35 mg/kg Hypnomidate® (Janssen Pharmaceutica, Beerse, Belgium) was applied to the mice for anesthetization and the skin and peritoneum were laterally incised to exteriorize the spleen. During surgery HCT116/CMVp-Luc cells, resuspended in PBS, were kept on ice. After repositioning of the spleen, the peritoneum was closed with Surgicryl® absorbable suture and the skin was clamped twice.

2.13.2 *In vivo* drug application

Intrasplenically transplanted SCID-beige mice were randomly assigned to 3 groups of 10 animals each. Oral application with daily doses of either solvent (10% Kolliphor in 0.9% NaCl) or 13 mg/kg body weight of Fluvastatin or Atorvastatin (in 0.9% NaCl) was started 24 h after transplantation. This dose agrees with a human equivalent dose of approximately 1 mg/kg body weight which is commonly applied in standard blood lipid lowering therapy [316,317]. Due to ethical reasons mice were sacrificed at day 28.

2.13.3 *In vivo* bioluminescence imaging

For monitoring of tumor growth and metastasis formation the bioluminescence imaging system NightOWL LB 981 (Berthold Technologies, Bad Wildbad, Germany) was used. Here, 150 mg/kg D-luciferin (Biosynth, Staad, Switzerland) was intraperitoneally applied to anesthetized (isoflurane gas) mice. Tumor growth as well as metastasis formation were imaged and quantified by WinLight (Berthold Technologies) and ImageJ (version 1.51j8, National Institutes of Health, USA). After termination of the experiment, spleen (as the transplantation site) and liver (as a metastasis target organ) were removed and shock frozen in liquid nitrogen for preparation of cryosections and further analysis (2.13.4 and 2.13.5).

2.13.4 MACC1 expression in xenograft tissue

The frozen tumor tissue was grinded to powder under liquid nitrogen. After adding of RL buffer and 10 pulse sonications at 40% for proper cell lysis, RNA isolation and qRT-PCR was carried out as previously described (2.3).

2.15 Statistical analysis

Microsoft Excel 2010 and GraphPad PRISM version 6.0 were used to perform calculations and statistical analysis. The comparison of two different groups was done by t-test whereas three or more different groups were compared by One-way or Two-way analysis of variance (ANOVA) and Dunnett, Turkey´s or Sidak´s post multiple comparison test. All significance tests were two sided with a confidence interval of 95% (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

3. Results

In this study we aimed to elucidate new strategies to intervene in tumor progression and metastasis formation via the novel target MACC1. On the one hand, we made use of the previously determined MACC1 promoter to identify new and possibly more potent transcriptional inhibitors. Therefore, we employed high-throughput drug screening and validated the identified inhibitors *in vitro* and *in vivo* for their effect and specificity on MACC1 expression and function (3.1). On the other hand, we wanted to illuminate the MACC1 signaling landscape to reveal additional drug intervention points. For this, we used a mass-spectrometry based approach to find interactors of tyrosine-phosphorylated MACC1. We further evaluated these for their functional impact on MACC1 signaling using a variety of cell-based assays (3.2).

3.1 Transcriptional inhibition of MACC1

3.1.1 HTS identifies statins as most potent inhibitors of MACC1 expression

Previously, our group identified the MACC1 promoter and, subsequently, Lovastatin as the first transcriptional inhibitor of MACC1 [238,274]. Now, we intended to reveal novel and possibly more potent transcriptional inhibitors of MACC1 by using an additional, independent HTS including different compound libraries: The Microsource, NIH and Prestwick libraries comprise 4241 compounds. The HTS was performed in cooperation with European Molecular Biology Laboratory (EMBL) and German Cancer Research Center (DKFZ), respectively. We used HCT116 cells expressing the luciferase reporter gene under control of the MACC1 promoter (HCT116/MACC1p-Luc) which is schematically represented in figure 12A. In the primary screen two doses (1 µM and 0.1 µM for NIH and Microsource library; 5 µg/ml and 0.5 µg/ml for Prestwick library) were applied to the cells and promoter activity was measured via luciferase expression and resulting luminescence intensity after addition of substrate. This process yielded 35 compounds able to reduce luciferase activity. These compounds were assessed in a counter selectivity screen using HCT116/CMVp-Luc cells to eliminate non-specific luciferase inhibitors. Additionally, we determined cytotoxicity of these compounds by MTT and ATP measurements for 24 h and 72 h, respectively. Based on the largest ratio between 72 h cytotoxicity and promoter activity reduction, four compounds (Fluvastatin, Bosutinib, PD173952, PD161570) were chosen for *in vitro* validation (Fig. 12C): cytotoxicity as well as reduction of MACC1 mRNA and protein expression was assessed in HCT116 wild-type cells for these four compounds. Here, we identified Fluvastatin as most effective inhibitor of MACC1 expression. The workflow of the HTS is depicted in figure 12B.

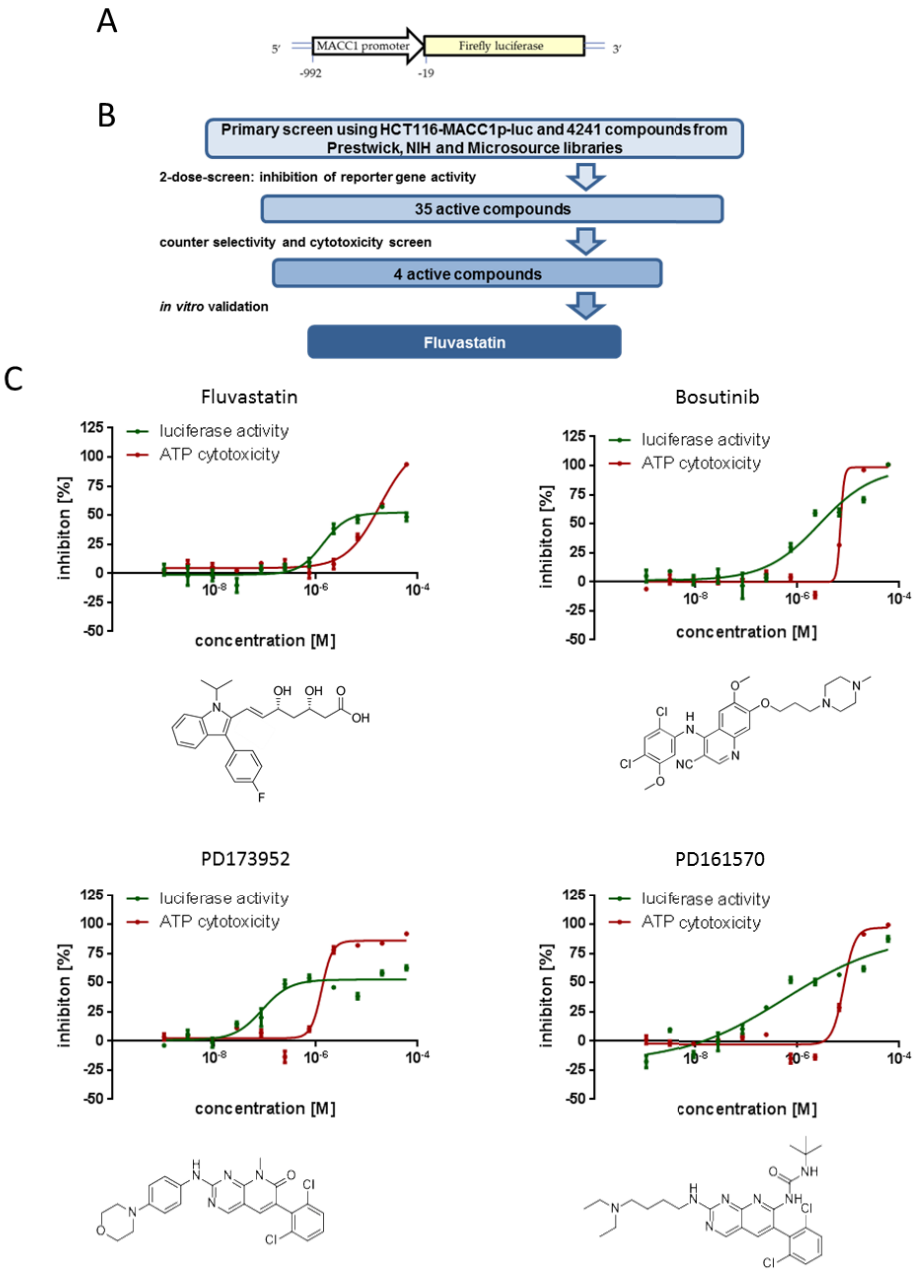


Fig. 12: HTS identifies statins as most potent transcriptional inhibitors of MACC1. (A) Scheme of construct expressed in HCT116/MACC1p-Luc cells used for HTS. The luciferase reporter gene expression is controlled by the MACC1 promoter (MACC1p). **(B)** Overview of stepwise identification of Fluvastatin as transcriptional inhibitor of MACC1 from 4241 Prestwick, NIH and Microsource library compounds via HTS and *in vitro* validation. **(C)** Dose-response curves and chemical structures of the four compounds showing best effects on MACC1 promoter activity: Fluvastatin, Bosutinib, PD173952 and PD161570. The four compounds were selected based on the largest ratio between Inhibition of luciferase activity (green) reflecting promoter activity after 24 h and cytotoxicity (red) determined by ATP measurement after 72 h of treatment. Luminescence intensities of promoter activity and cytotoxicity assay were normalized to respective solvent controls (0.3% DMSO). Results represent means + Standard error of the mean (SEM) of three independent experiments.

Previously, we identified Lovastatin, another member of the statin family, as transcriptional inhibitor of MACC1 [274]. This prompted us to evaluate all clinically employed statins (Fluvastatin, Atorvastatin, Lovastatin, Pitavastatin, Simvastatin, Rosuvastatin, Pravastatin) on their ability to reduce MACC1 expression. Indeed, we showed that most of them reduce MACC1 expression on mRNA and protein level in a concentration-dependent manner (1-10 µM) in HCT116 cells. Fluvastatin (Fig. 13A) treatment at 1 µM reduced MACC1 expression below 50% compared to the control treated cells whereas Lovastatin (Fig. 13B), Atorvastatin (Fig. 13C) and Pitavastatin (Fig. 13D) reached this only at 2.5 µM and Simvastatin at 5 µM (Fig. 13E). Fluvastatin, Lovastatin and Atorvastatin treatment showed a maximal inhibition of MACC1 at 7.5 µM with approximately 10% remaining expression. Comparably, Simvastatin and Pitavastatin reduced MACC1 expression to about 30% and 40%, respectively.

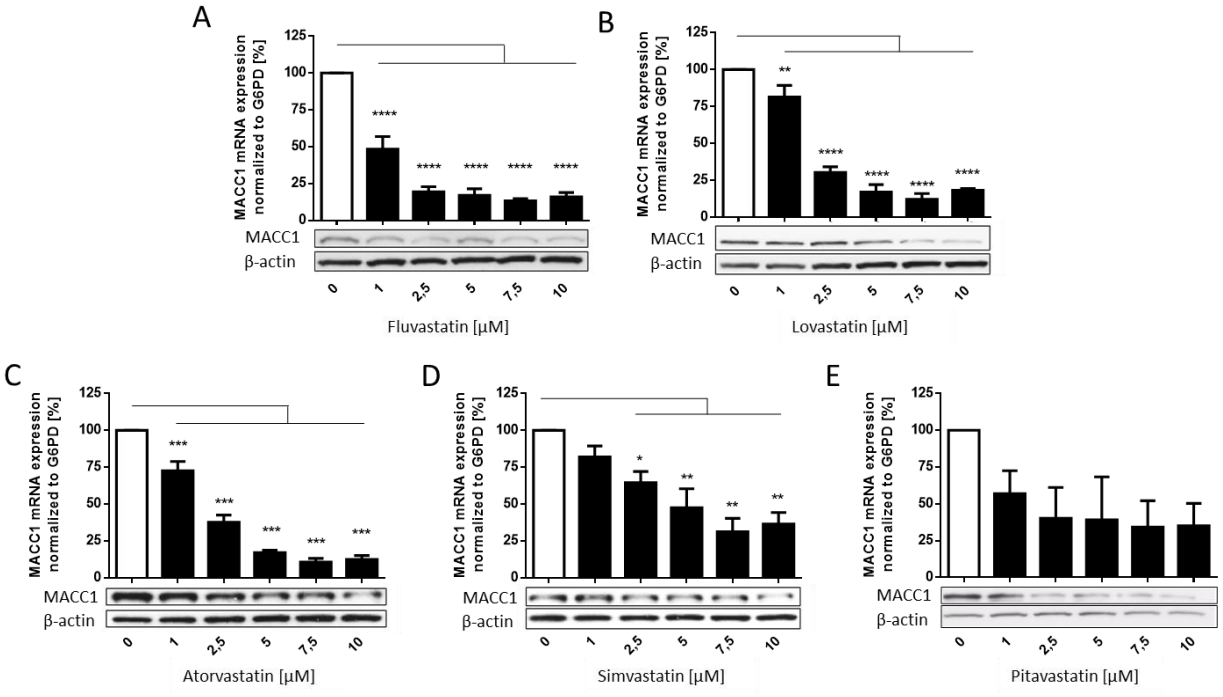


Fig. 13: Different members of the statin family reduce MACC1 expression. MACC1 showed a dose-dependent (0 -10 µM) decrease of mRNA and protein expression upon 24 h treatment with Fluvastatin **(A)**, Lovastatin **(B)**, Atorvastatin **(C)**, Simvastatin **(D)** and Pitavastatin **(E)**. MACC1 mRNA levels were normalized to G6PD mRNA expression and respective solvent controls (0.1% DMSO, white bars). Results for mRNA represent means + Standard error of the mean (SEM) of three independent experiments and for WB one representative example of at least two independent experiments is shown. In the WB, β-actin served as loading control. Significant results were determined by one-way ANOVA and Dunnett's multiple comparison test with a confidence interval of 95% (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

3.1.2 Statins reduce endogenous MACC1 expression in different cancer entities

In order to further validate our previous findings, we examined the statins in additional CRC and other cancer entity wild-type cell lines. We have chosen Fluvastatin and Atorvastatin as most effective statins for representative verification.

Both statins were able to reduce MACC1 expression on mRNA and protein level in the CRC cell lines SW48 and HT29 in a concentration-dependent manner. Compared to solvent control, Fluvastatin treatment decreased MACC1 expression to approximately 50% at 5 µM in SW48 and 10 µM in HT29, whereas Atorvastatin diminished it to approximately 60% in SW48 and 70% in HT29 at 10 µM (Fig. 14A). Furthermore, both statins decreased MACC1 mRNA and protein expression in a pancreatic (BxPC3) and a gastric cancer cell line (MKN45). In BxPC3 cells Fluvastatin treatment reached over 50% reduction at 5 µM and about 75% at 10 µM whereas Atorvastatin treatment reduced MACC1 expression under 50% at 10 µM (Fig. 14B). Both statins reduced MACC1 expression to about 70% in MKN45 cells with the highest concentration tested (10 µM). These results are depicted in figure 14C.

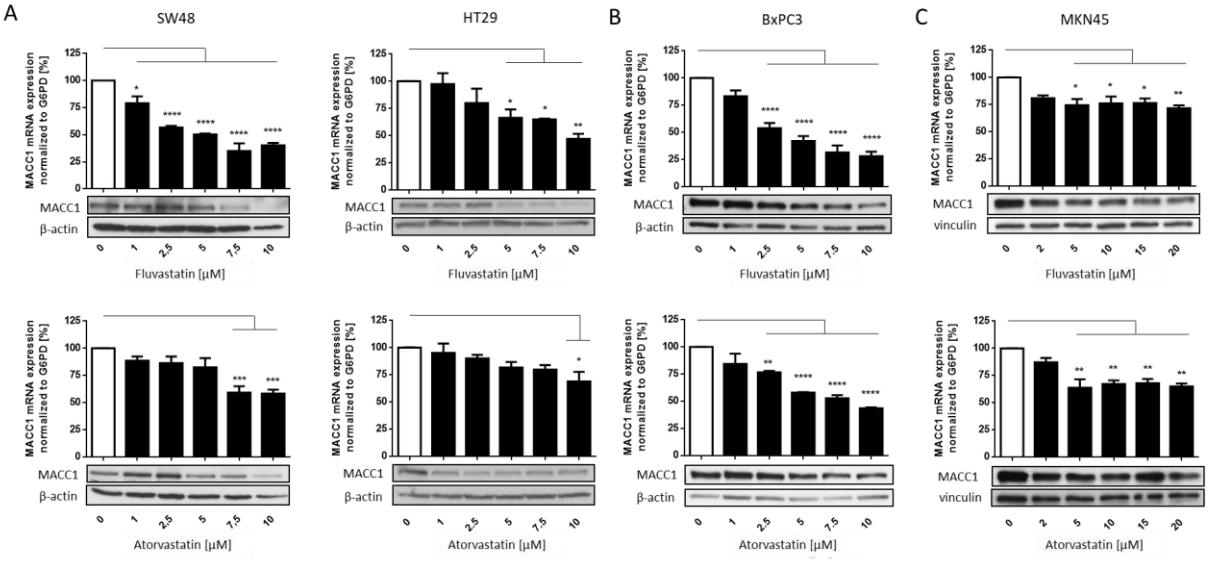


Fig. 14: Statins reduce endogenous MACC1 expression in different cancer entities. (A) Dose-dependent reduction of MACC1 mRNA and protein expression by Fluvastatin and Atorvastatin (0 – 10 µM) in further CRC cell lines SW48 and HT29. MACC1 mRNA and protein expression was also diminished by Fluvastatin and Atorvastatin in BxPC3 (pancreatic cancer) **(B)** and MKN45 (gastric cancer) cells **(C)**. MACC1 mRNA levels were normalized to G6PD mRNA expression and respective solvent controls (0.1% DMSO, white bars). Results for mRNA represent means + SEM of three independent experiments and for WB one representative example of three independent experiments is shown. In the WB, β-actin or vinculin served as loading control. Significant results were determined by one-way ANOVA and Dunnett's multiple comparison test with a confidence interval of 95% (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

3.1.3 Statins reduce endogenous MACC1 expression in different CRC cell models

For later functional and specificity analysis of the statin effect on MACC1, genetically modified models of HCT116 were generated. We chose models of HCT116 cells stably overexpressing MACC1-GFP, a MACC1-inducible overexpressing cell line and HCT116 with MACC1 knock-out (HCT116/MACC1 -/-) (see 2.2). First, it was of importance to evaluate statin treatment on MACC1 expression in these models. Figure 15A shows the statin effect on HCT116 cells overexpressing either GFP or a MACC1-GFP fusion protein. Both, Fluvastatin and Atorvastatin, diminished MACC1 mRNA expression in HCT116/GFP cells. The mRNA levels remained stable in HCT116/MACC1-GFP under Fluvastatin treatment and were not significantly decreased under Atorvastatin treatment. In more detail, the Western blot analysis demonstrates that only the endogenous, MACC1 promoter-driven MACC1 expression is reduced by statin treatment with a concomitant, strong overexpression of MACC1-GFP.

To induce MACC1 expression in HCT116/MACC1, cells were treated with doxycycline (+Dox) or solvent H_2O (-Dox) for 48 h. As seen in figure 15B, MACC1 mRNA expression was significantly increased (about 30%) in the control cells (0 µM), which was also confirmed in the WB. In both, doxycycline treated and untreated cells, statin treatment led to a decrease in MACC1 mRNA and protein expression while the +Dox cells retained a higher level compared to -Dox cells. For instance, under Fluvastatin treatment +Dox cells showed mRNA expression levels of approximately 85% at 1 µM and 70% at 2.5 µM compared to 60% and 45% in -Dox cells, respectively.

HCT116/Cas9 also reacted to Fluvastatin treatment: Figure 15C demonstrates a reduction of MACC1 mRNA expression to about 70% and 60% at 1 µM and 2.5 µM confirmed by protein expression via WB. In HCT116/MACC1 -/- cells, MACC1 expression was not abundant in qRT-PCR or WB.

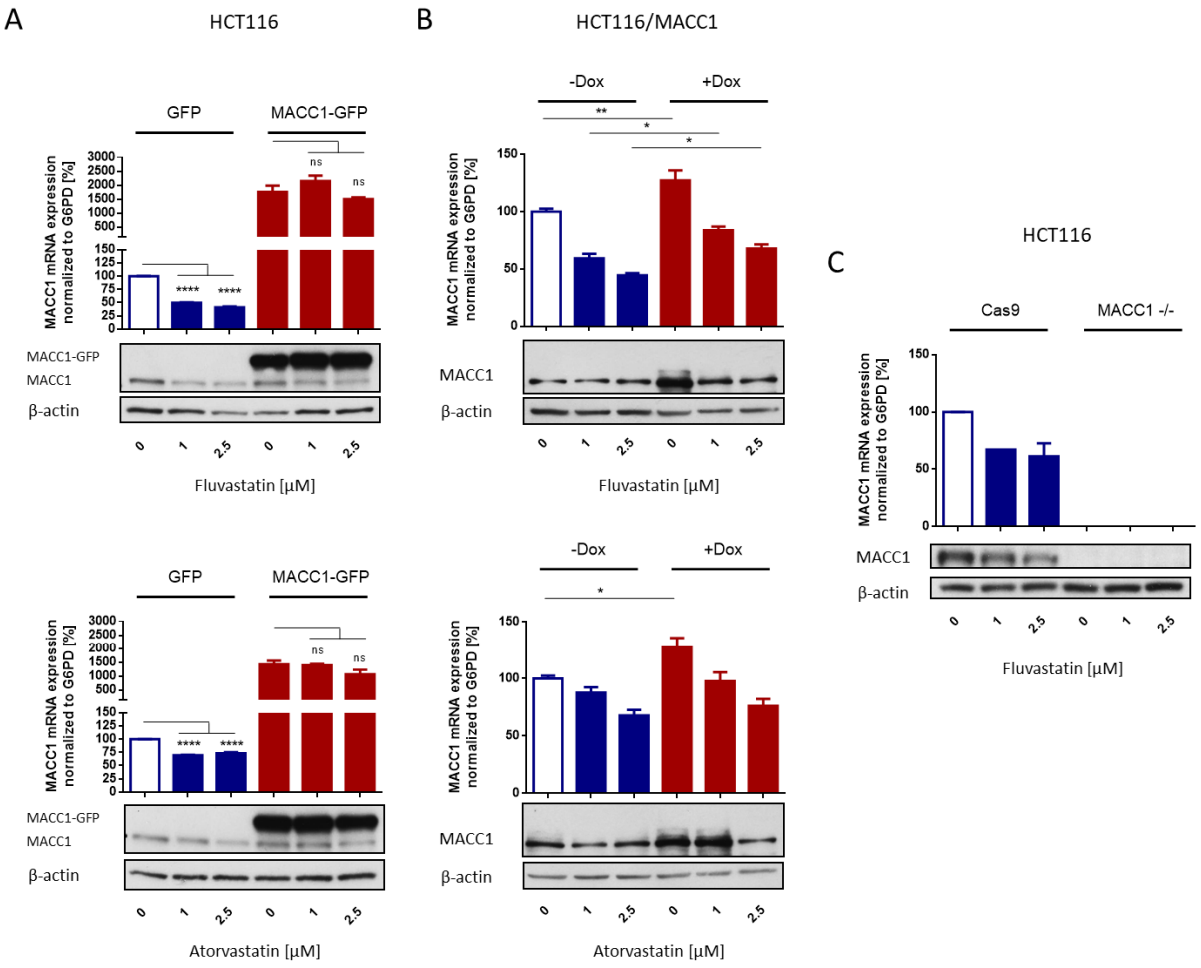


Fig. 15: Statins reduce endogenous MACC1 expression in different CRC cell models. Different cell models modulated in MACC1 expression were generated and tested for reactivity to statin treatment. **(A)** HCT116 cells stably overexpressing GFP show a reduction of MACC1 mRNA and protein expression upon Fluvastatin and Atorvastatin treatment (1 or 2.5 µM). HCT116/MACC1-GFP cells present stable expression of MACC1 mRNA after statin treatment while only endogenous MACC1 protein levels are diminished by statins. **(B)** HCT116 cells with doxycycline inducible MACC1 expression are affected by statin treatment regardless of MACC1 induction, but induced cells (for 48 h; +Dox) show a generally higher expression of MACC1 mRNA or protein than those not induced (-Dox). **(C)** In HCT116 cells transduced with a Cas9 plasmid serving as control cell line, Fluvastatin treatment led to a decrease of MACC1 mRNA and protein expression. For the respective MACC1-/- cell line, MACC1 expression was absent in qRT-PCR and WB. MACC1 mRNA levels were normalized to G6PD mRNA expression and respective treatment controls (DMSO, H_2O; white bars). Results for mRNA represent means + SEM of at least two independent experiments and for WB one representative example of two independent experiments is shown. In the WB, β-actin served as loading control. Significant results were determined by one-way ANOVA and Dunnet´s or Sidak´s multiple comparison test with a confidence interval of 95% (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

3.1.4 Statins specifically inhibit MACC1-mediated functions *in vitro*

MACC1 is known to induce a variety of cellular functions involved in tumor progression and metastasis formation, such as proliferation, colony formation and motility [100]. To evaluate the potential of statins for specific inhibition of MACC1-mediated cellular functions, proliferation and clonogenic assays were performed. For proliferation assessment, 72 h live imaging with subsequent confluence analysis was used on HCT116/MACC1 cells (-Dox/+Dox) treated with 1 µM Fluvastatin or 2.5 µM Atorvastatin. Figure 16A+B show the relative proliferation determined by area under the curve (AUC) of the respective growth curves normalized to solvent treated controls (white bars). MACC1 overexpression in +Dox cells exerted a proliferative phenotype demonstrated by an approximate 30% increase in cell growth compared to -Dox cells. Proliferation was reduced to 40% by Fluvastatin and to 55% by Atorvastatin treatment which could be rescued by MACC1 induction to 77% and 93%, respectively (Fig. 16A).

In a similar manner, HCT116/Cas9 and HCT116/MACC1 -/- cells were subjected to the proliferation assay. As depicted in figure 16B, MACC1 knock-out led to an over 50% decrease in cell growth compared to Cas9 control cells. Interestingly, we found a dose-dependent reduction in the proliferation of HCT116/Cas9 under Fluvastatin treatment whereas the MACC1 knock-out cells remained unaffected.

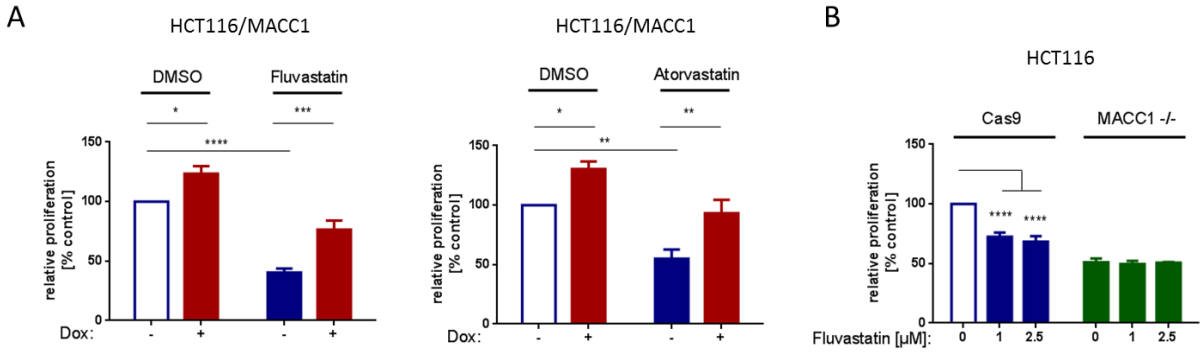


Fig. 16: Statins specifically inhibit MACC1-mediated proliferation. Relative proliferation was determined with the IncuCyte® live imaging system for 72 h and calculated by the AUC normalized to solvent controls (0.1% DMSO; white bars). **(A)** HCT116 cells with doxycycline induced MACC1 expression (+Dox) demonstrated a 30% increase in proliferation. MACC1-induced proliferation in +Dox compared to -Dox cells was still observed under statin treatment indicating a MACC1-specific rescue of proliferative function. **(B)** Confirmation of the previous findings with MACC1 knock-out cells (HCT116/MACC1 -/-). MACC1 knock-out resulted in an over 50% reduction in proliferation compared to control cells (HCT116/Cas9). Control cell proliferation was decreased by Fluvastatin treatment (1 and 2.5 µM) whereas HCT116/MACC1 -/- cells remained unaffected. Results represent means + SEM of at least three independent experiments. Significant results were determined by two-way ANOVA and Sidak´s multiple comparison test with a confidence interval of 95% (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

The clonogenic assay was performed to investigate the reproductive viability. In principle, every individual cell is challenged for its ability to divide limitlessly which is judged by their colony forming potential [305-307]. Here, it was employed to assess MACC1-dependent reproductive viability upon statin treatment. This was used to evaluate the specificity of the statin effect on MACC1-mediated functions. Low density seeding of HCT116/GFP or HCT116/MACC1-GFP cells (400 cells/6-well) was followed by treatment with 1 µM and 2.5 µM of statin for seven days. The ability to form and grow colonies was determined by imaging and analyzing the fixed and stained cells (Fig. 17A). Here, the number of colonies (Fig. 17B) and colony covered area (Fig. 17C) were measured with ImageJ (version 1.51j8, National Institutes of Health, USA). By normalizing the values of treated cells to solvent controls of each respective cell line (HCT116/GFP or HCT116/MACC1-GFP), adjusted rescue values were calculated (Fig. 17C).

In general, solvent treated cells overexpressing MACC1-GFP showed an approximately 1.5 to 2-fold increase in colony covered area and colony number compared to control cells. Upon statin treatment colony formation was reduced in HCT116/GFP cells in a concentration-dependent manner whereas the overexpression of MACC1-GFP rescued this effect. Especially, the treatment with 1 µM of either statin, reducing control cell colony formation, hardly affected colony number (Fig. 17B) or colony covered area (Fig. 17C) of MACC1-GFP overexpressing cells. When treated with 2.5 µM of statin, MACC1-GFP overexpression still partly rescued colony formation. This is also represented by the adjusted rescue values: Colony number was rescued by 16% (1 µM) and 14% (2.5 µM) upon Fluvastatin treatment or 9% (1 µM) and 20% (2.5 µM) upon Atorvastatin treatment. Adjusted values showed a rescue of colony covered area by 30% (1 µM) or 9% (2.5 µM) for Fluvastatin and 20% (1 µM) or 25% (2.5 µM) for Atorvastatin treatment.

In general, the rescue effects by forced MACC1 overexpression demonstrated in figure 16 and 17 indicate a specific effect of statins on MACC1 function, particularly clonogenicity and proliferation, respectively.

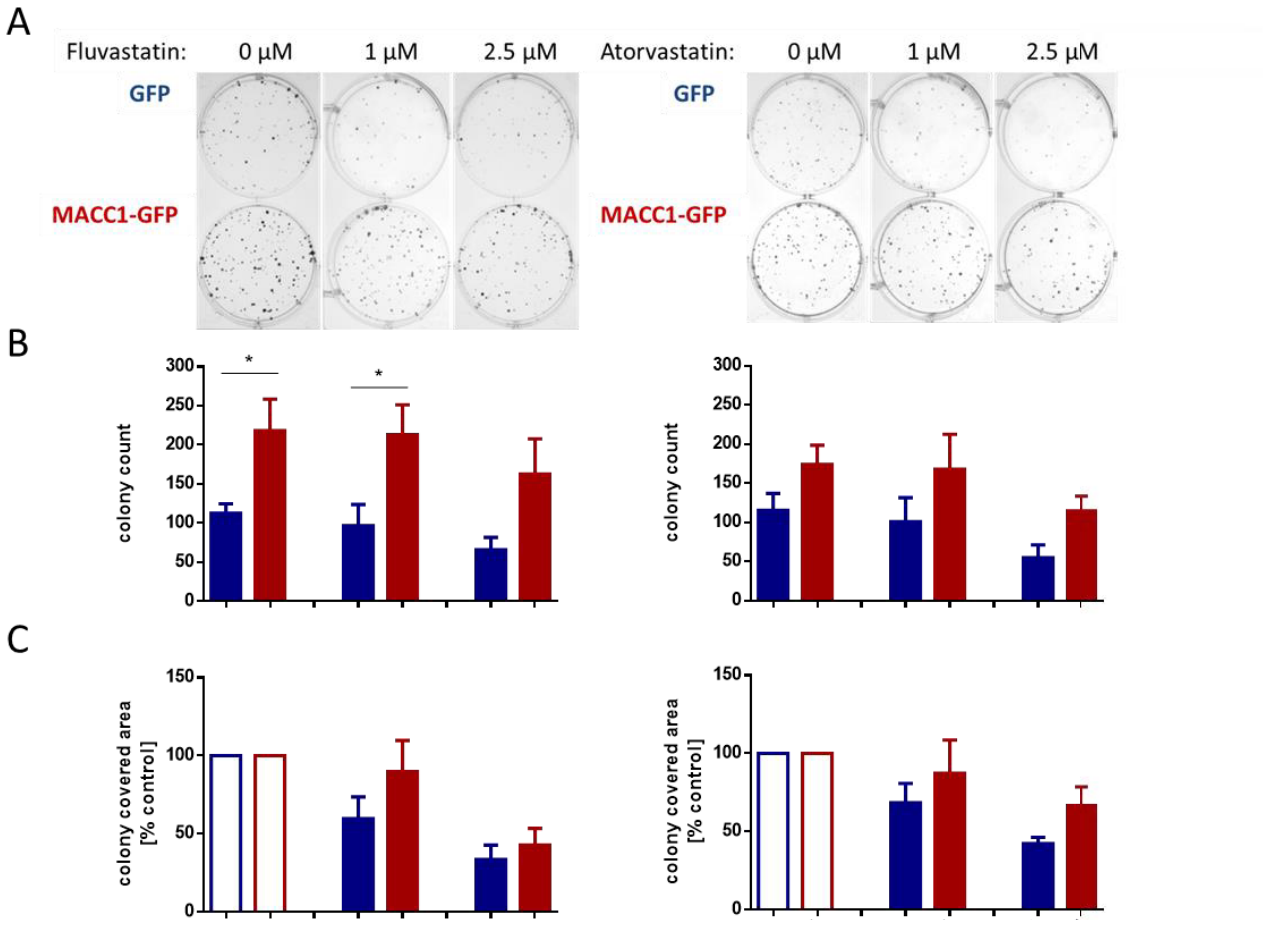


Fig. 17: Statins specifically inhibit MACC1-mediated clonogenicity. Clonogenicity of formaldehyde fixed and crystal violet stained cells was imaged **(A)** and assessed by colony number **(B)** and the colony covered area **(C)**. Stable overexpression of MACC1-GFP in HCT116 cells led to a strongly augmented (1.5 to 2-fold) colony formation compared to HCT116/GFP cells. Colony formation of control cells was reduced upon treatment with 1 µM of either statin whereas MACC1-GFP overexpressing cells remained nearly unaffected indicating a MACC1-specific rescue of clonogenic function. A partial rescue was also seen upon treatment with 2.5 µM of either statin. Results represent means + SEM of three independent experiments. One representative of nine technical replicates divided in three independent experiments is shown. Significant results were determined by two-way ANOVA and Sidak´s multiple comparison test with a confidence interval of 95% (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

3.1.5 Statin treatment decreases tumor burden and metastasis formation *in vivo*

As shown previously, MACC1 aggravates tumor growth and induces metastasis formation. Both were specifically reduced by RNAi or use of previously identified MACC1 inhibitors Lovastatin and Rottlerin [100,219,222,225,233,237,240,250,274]. Therefore, we wanted to proof this concept for Fluvastatin and Atorvastatin *in vivo*. In collaboration with the EPO Berlin-Buch GmbH, SCID-beige mice were intrasplenically transplanted with $3x10^5$ HCT116/CMVp-Luc cells and treated orally with either solvent or daily doses of 13 mg/kg body weight of the respective statin (10 animals per group). This corresponds to a human equivalent dose of approximately 1 mg/kg body weight which is commonly used in statin therapy for blood lipid reduction [316,317]. Tumor growth and metastasis formation were continuously monitored by bioluminescence imaging over 24 days until the ethical end point (cancer burden of control group animals) was reached. One animal had to be sacrificed early (day 19) due to this reason.

Bioluminescent signals of all mice in lateral view at day 24 are presented in figure 18A. The lateral view captures the bioluminescent signals emitted by the tumor cells growing in the spleen forming the primary tumor. In general, most control animals show larger signal areas and stronger signal intensities than statin treated ones judged by the luminescence intensity scale. This was confirmed by the integrated luminescence quantification with ImageJ (version 1.51j8, National Institutes of Health, USA) shown as means + SEM per group in figure 18B. These data demonstrate that treatment with either statin led to a strong restriction of tumor growth. Here, significant differences ($p = 0.013$) in tumor growth were already observed between control and Atorvastatin treated animals at day 15. This effect increased over time presenting strong differences between control and statin treated animals at day 19 (Fluvastatin and Atorvastatin: $p < 0.0001$) and day 24 (Fluvastatin: $p < 0.0001$, Atorvastatin: $p = 0.0002$).

For the assessment of health or imminent death, progressive loss of body weight represents a major criterion for *in vivo* studies with mice [318]. In accordance with this, body weight of mice was monitored besides the bioluminescent imaging. Until the termination of the experiment, neither control group nor statin treated animals displayed significant changes or differences between groups in body weight (Fig. 18C).

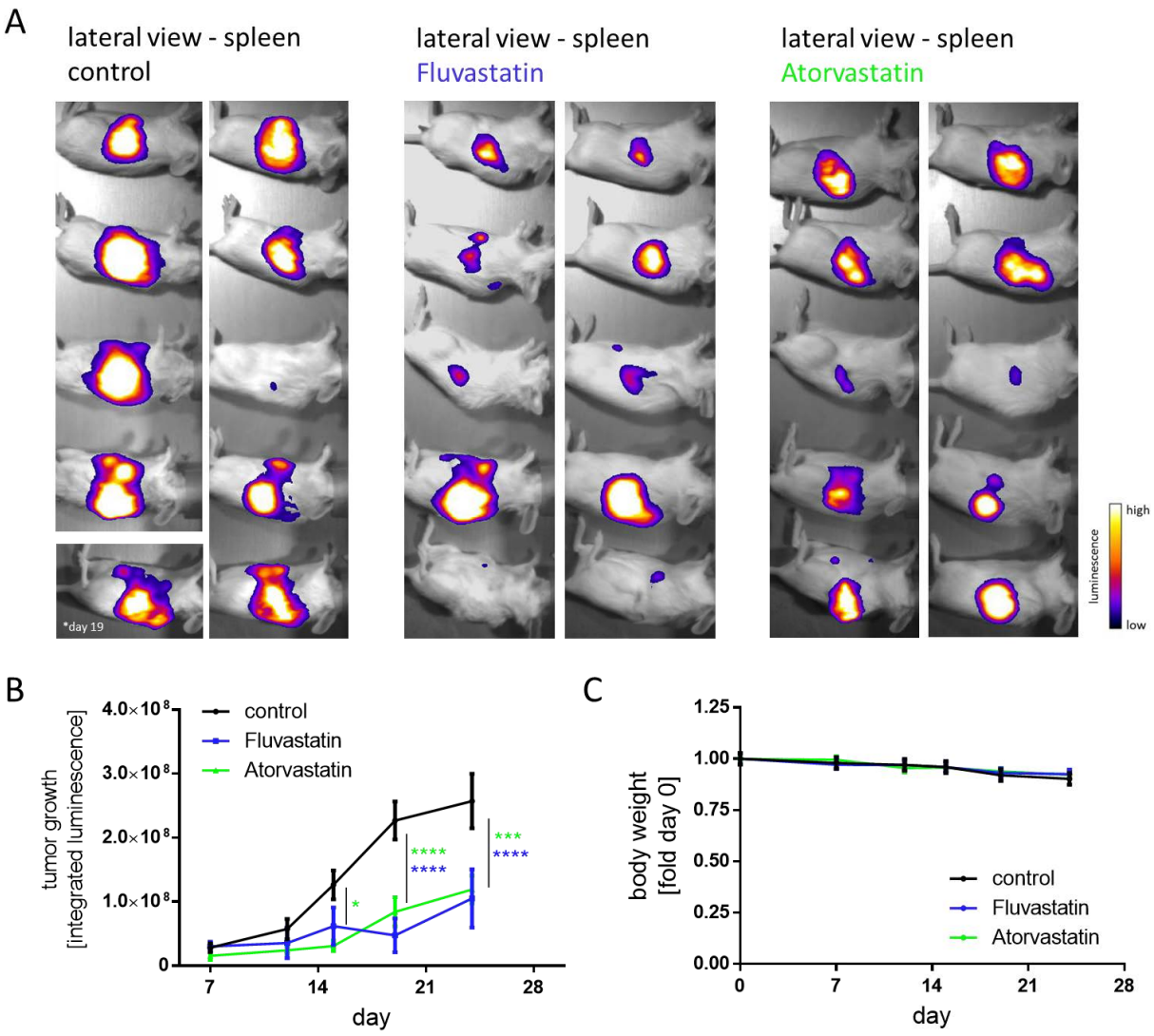


Fig. 18: Statin treatment decreases tumor burden *in vivo*. (A) Bioluminescent imaging at day 24 of SCID-beige mice from lateral view to detect tumor growth in the spleen. One animal in the control group was sacrificed due to ethical reasons already on day 19. In general, Fluvastatin and Atorvastatin treated animals show smaller signal areas and weaker signals indicating restricted tumor growth. **(B)** Bioluminescence intensity was quantified via ImageJ and averaged per group and day. These data demonstrate a significant restriction of tumor growth through statin treatment. **(C)** Body weight of the mice was regularly monitored as indicator of health or imminent death. None of the animals displayed drastic changes in body weight. Results represent means + SEM of 10 animals per group. Significant results were determined by two-way ANOVA and Dunnet´s multiple comparison test with a confidence interval of 95% (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

Bioluminescent signals of all mice in ventral view at day 24 are presented in figure 19A. Imaging from the ventral view determines the bioluminescent signals emitted by tumor cells forming metastasis in the liver. In general, most control animals show larger signal areas and stronger signal intensities than statin treated ones judged by the luminescence intensity scale. This was confirmed by the integrated luminescence quantification with ImageJ (version 1.51j8, National Institutes of Health, USA) shown as means + SEM per group in figure 19B. These data demonstrate that treatment with either statin led to a strong restriction of metastasis formation. At day 19 and 24 significant differences in metastasis formation were observed between control and statin treated animals: day 19 (Fluvastatin: $p < 0.0001$, Atorvastatin: $p = 0.0001$) and day 24 (Fluvastatin: $p < 0.0001$, Atorvastatin: $p < 0.0001$). Moreover, we found reduced amounts of MACC1 transcripts in the livers of statin treated mice reflecting their MACC1 specific targeting also in an *in vivo* setting (Fig. 19C).

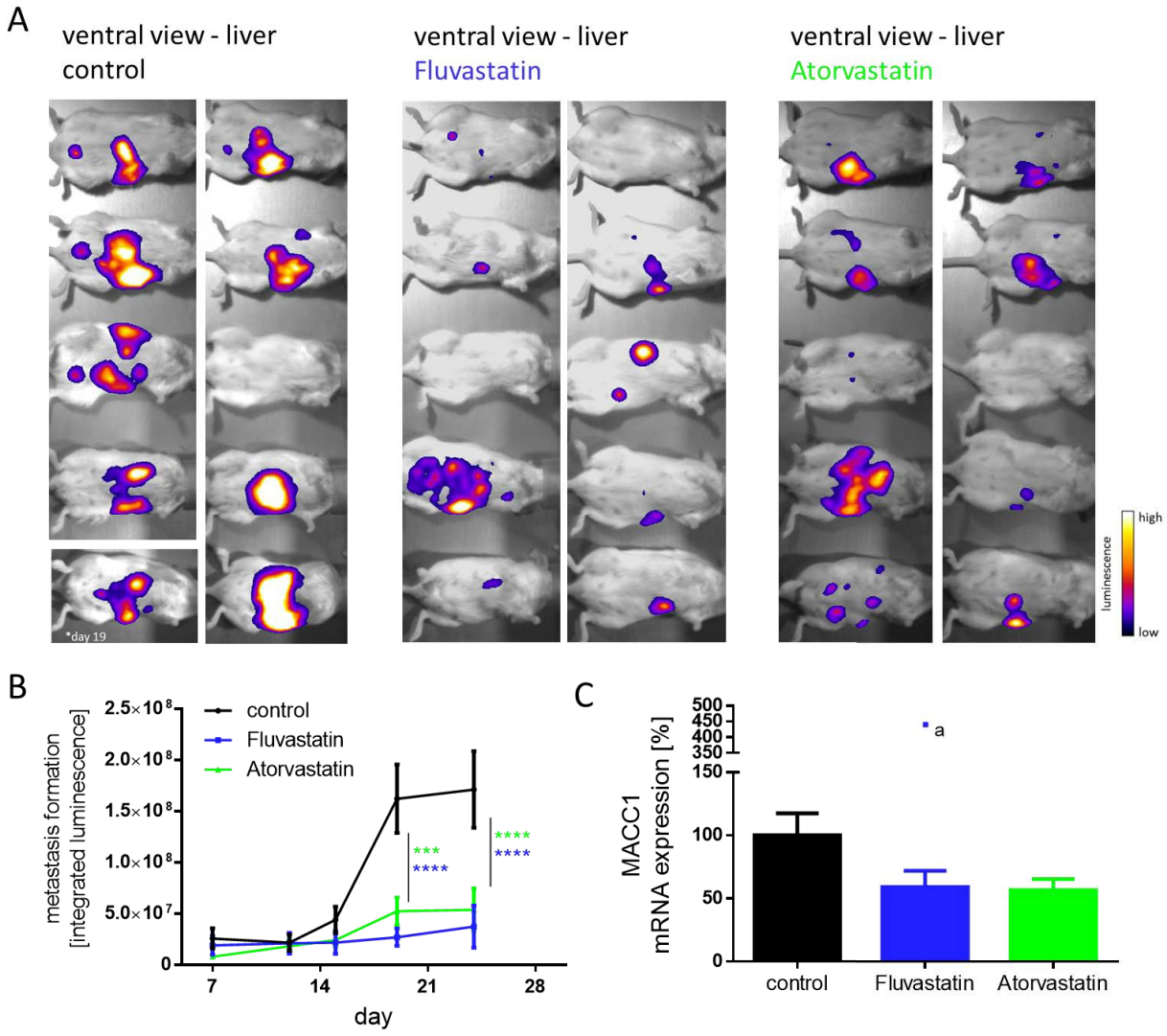


Fig. 19: Statin treatment decreases metastasis formation *in vivo*. (A) Bioluminescent imaging at day 24 of SCID-beige mice from ventral view to detect metastasis formation in the liver. One animal in the control group was sacrificed due to ethical

reasons already on day 19. In general, Fluvastatin and Atorvastatin treated animals show smaller signal areas and weaker signals indicating reduced metastasis formation. **(B)** Bioluminescence intensity was quantified via ImageJ and averaged per group and day. These data demonstrate a significant restriction of metastasis formation through statin treatment. **(C)** Fluvastatin and Atorvastatin treated mice further showed reduced amounts of MACC1 transcripts in the liver compared to control animals. Results represent means + SEM of 10 animals per group. Significant results were determined by two-way ANOVA and Dunnet´s multiple comparison test with a confidence interval of 95% (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$). a – one outlier was identified by the ROUT method (Q=1%) and Grubbs´s test (alpha=0.01) which was not considered for the calculation of the mean MACC1 mRNA expression in the livers.

In summary, we identified Fluvastatin as additional transcriptional inhibitor of MACC1 via HTS. Together with our previous results, this prompted us to test all clinically employed statins for their inhibitory potential on MACC1 expression. Indeed, we demonstrated that most statins reduce MACC1 expression on mRNA and protein level in CRC cells. Fluvastatin and Atorvastatin, selected as strongest inhibitors, diminished MACC1 mRNA and protein expression also in pancreatic and gastric cancer cells. Both statins were further shown to specifically inhibit MACC1-dependent cell functions such as proliferation and colony formation. We proved this by partially rescuing the inhibitory effect by inducible or stable overexpression of MACC1 in CRC cells. Finally, both statins decreased tumor growth and metastasis formation as well as MACC1 transcripts in the liver of a xenografted mouse model for CRC metastasis.

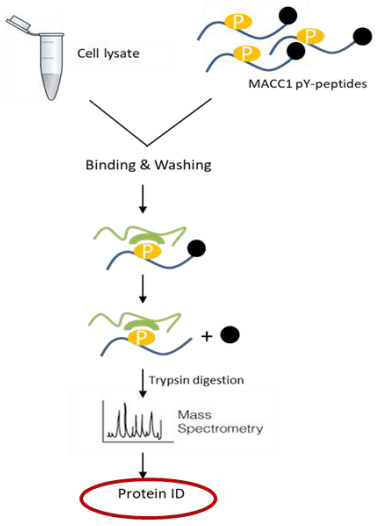
3.2 MACC1 phospho-interactome and signaling landscape

3.2.1 A MS-based screen identifies interactors of tyrosine phosphorylated MACC1

The domain architecture of MACC1 comprises several distinct domains such as ZU5, UPA, SH3 and two DDs indicating its involvement in protein-protein interaction or protein recruitment [100,290-295]. MACC1 also contains further protein interaction motifs such as the clathrin box, NPF and DPF tripeptides as well as proline-rich sequences (PxPxP, KxxPxxP) and several serine (Ser, S), threonine (Thr, T) or tyrosine (Tyr, Y) sites accessible for phosphorylation [100]. Especially tyrosine phosphorylation represents a crucial mechanistic switch in signal transduction. Phosphorylation of specific Y-sites mostly leads to an activation of the respective protein. Phosphorylated tyrosines (pY), additionally, serve as docking sites for SH2 domain containing proteins, thereby fostering downstream signaling [296-299]. Therefore, we aimed to identify pY-dependent interactions of MACC1 in CRC.

In cooperation with the Küster lab at TUM, we performed pull-down experiments in SW480 and SW620 CRC cells as well as placenta tissue. Placenta tissue is an easily accessible human primary tissue expressing a large variety of proteins: 69% of all human proteins were found to be expressed in placenta tissue compared to a 44% expression baseline detected in most other tissues [319-321]. Therefore, placenta served as control tissue. Pull-downs were carried out with bead-coupled peptides representing all potential pY-sites of MACC1. Subsequent MS revealed the interaction with several proteins via specific pY-sites on MACC1 (Fig. 20A). An overview of pulled-down proteins is given in figure 20B. For further analysis, we focused on proteins identified in both cell lines and both conducted screens. Most interestingly, some of these were found to be involved in RTK signalosome formation: GRB2, SHP2, SHC1, STAT5B and PLCG1 (Fig. 20C) [42].

A



B

	pull-down in			
protein	SW480	SW620	human placenta lysate	receptor tyrosine complex
GRB2	+	+	+	+
SHP2	+	+	+	+
STAT5B	+	+	+	+
SHC1	+	+	+	+
PLCG1	+	+	+	+
ECHB	+	+	+	
ECHA	+	+	+	
LANCL1	+	+	+	
DECR	+		+	
PTPN6	+	+	+	
RUVBL1	+	+	+	
TNS4	+		+	
TRAF3	+		+	
RPUSD4	+		+	
PLCG2	+		+	
RUVBL2		+	+	
SHIP1		+	+	+
PIK3R1			+	+
LYN			+	+
CRK			+	+
RASA1			+	+

C

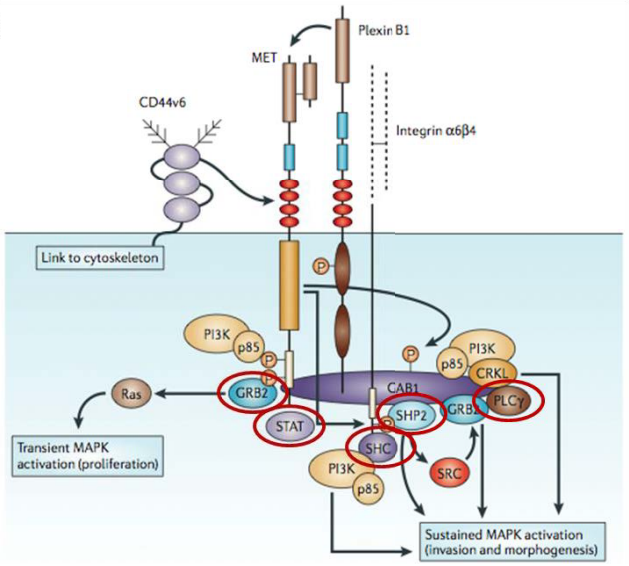


Fig. 20: A phosphoproteomic screen identifies interactors of tyrosine phosphorylated MACC1. (A) Scheme of assay workflow. Cell lysates were incubated with bead-coupled MACC1 pY-peptides. After binding and washing steps, the beads were removed, and pulled-down proteins trypsin digested. Subsequent MS revealed several potential interactors of pY-MACC1. **(B)** Tabular results of identified interactors of pY-MACC1. Pull-downs were performed in SW480 and SW620 CRC cells as well as in human placenta lysate as control tissue. The screen was conducted twice revealing overlapping bindings of pY-MACC1 with a variety of proteins. **(C)** Five proposed interactors are known to form signaling complexes below RTKs which is schematically represented here (modified from [42]). These five interactions with GRB2, SHP2, STAT5B, SHC1 and PLCG1 were subjected to further analysis. MACC1 – metastasis associated in colon cancer 1; MET - MET receptor tyrosine kinase; GRB2 - growth factor receptor-bound protein 2; RAS - rat sarcoma viral oncogene homolog; MAPK - mitogen-activated protein kinase; STAT - signal transducer and activator of transcription; GAB1 - GRB2-associated-binding protein 1; SHC - SHC-transforming protein ; SHP2 - SH2 domain-containing phosphatase 2; PI3K - phosphoinositide 3-kinases; p85 - p85 regulatory subunit of phosphoinositide 3-kinase; PLCγ – phospholipase C gamma; CRKL - Crk-like protein; SRC - non-receptor tyrosine kinase SRC; CD44v6 - cluster of differentiation 44 splice variant 6.

In order to validate the findings of the MS screen, we selected Co-IP experiments as one of the established standard methods for identification and verification of protein-protein interaction [322]. We performed pull-downs with specific antibodies for the respective interactors or MACC1 in SW480 cells with ectopic MACC1 overexpression (SW480/MACC1) (Fig. 21A) and SW620 wild-type cells (Fig. 21B). The interaction of MACC1 with all five suggested proteins was confirmed in WB using the antibody of the respective opposing interactor. Protein size was referenced either with an input of whole cell lysate or a positive control of the pull-down with the respective interactor antibody.

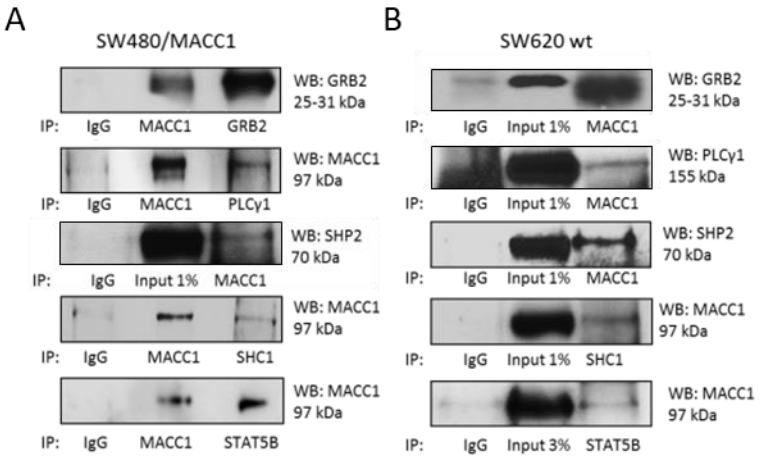


Fig. 21: Co-Immunoprecipitation (Co-IP) confirms interactions with suggested proteins. Co-IP was performed with cell lysates from SW480 CRC cells with ectopic MACC1 overexpression (SW480/MACC1) **(A)** and SW620 CRC wild-type cells **(B)**. All five suggested bindings of MACC1 with GRB2, PLCG1, SHP2, SHC1 and STAT5B were validated in this assay. Reference of protein size was achieved with an input of whole cell lysate or a positive control of the pull-down with the respective interactor antibody. Results show one representative example of at least two independent experiments.

In further pull-down experiments with subsequent MS, we wanted to confirm the specificity of the interactions for distinct pY-sites on MACC1. For this reason, we performed separate pull-downs with unphosphorylated and phosphorylated versions of the previously identified peptides. Figure 22 shows that four of the five interactors bind specifically to phosphorylated and selectively to one respective site on MACC1: pY365 – STAT5B; pY379 – GRB2 and SHP2; pY789 – SHC1. PLCG1 showed a more promiscuous binding pattern but favored the binding to pY379 and pY789 over their respective opposing peptide version. The results were identical for SW480 and SW620 cells except the binding of PLCG1 to Y365. Here, the cell lines demonstrate contradictory results.

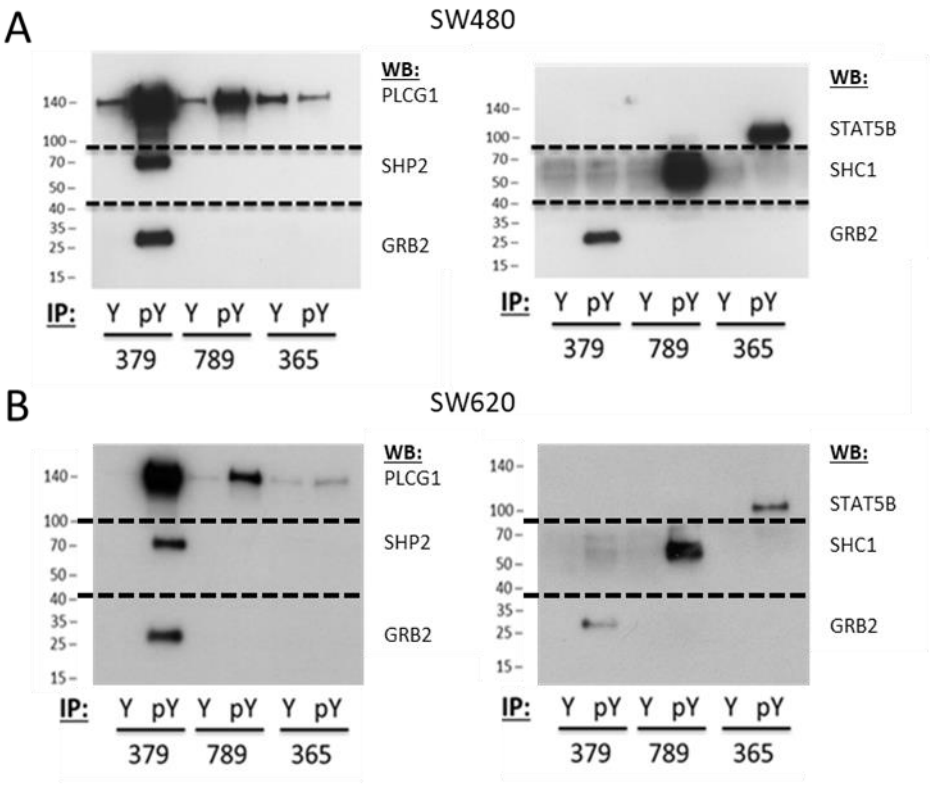


Fig. 22: Peptide pull-downs reveal selectivity of interactors for distinct pY-sites on MACC1. Phosphorylated or unphosphorylated peptides of the identified pY-interaction sites on MACC1 were used to pull-down proteins from lysates of SW480 **(A)** and SW620 wild-type cells **(B)**. Subsequent WB showed the selective binding of SHP2 and GRB2 to the phosphorylated Y379 peptide whereas SHC1 and STAT5B interacted with pY789 and pY365, respectively. PLCG1 binding was rather unspecific: It showed the strongest interaction with pY379 followed by pY789 and contradictory results for Y365.

3.2.2 pY-interaction sites are important for MACC1 function

To assess the functional relevance of the pY-interactions sites on MACC1, we performed site-directed mutagenesis. First, we focused on the interaction sites Y379 and Y789 which bind the majority of interactors: GRB2, SHP2, SHC1, PLCG1. All of these have been found to regulate cellular signaling such as the ERK pathway governing many decisive processes such as proliferation or migration [23,42,44-56].

Figure 23A shows the putative domain structure of MACC1 including mutated tyrosine sites as well as an exemplary histogram for the mutation at Y379. By a single nucleotide exchange from adenosine to thymidine, the tyrosine is substituted by a structural similar phenylalanine (F) which is not accessible for phosphorylation. Next, HCT116 CRC cells transduced either with GFP, MACC1-GFP or MACC1-GFP mutated at Y379 and Y789 (Y379F+Y789F) were employed in a Boyden chamber migration assay. Overexpression of MACC1-GFP led to a more than 2-fold increase in cell migration compared to GFP control cells. Interestingly, the cell clones harboring tyrosine-mutated MACC1 showed significantly reduced migratory ability compared to wild-type MACC1-GFP transfected clones (Fig. 23B). Figure 23C demonstrates

a similar effect on HGF-induced proliferation. After seeding, cells were accommodated for 24 h in 10% FBS medium and then switched to 0% FBS medium containing 20 ng/ml HGF. Via cell confluence assessment, an increased proliferative capacity of HCT116/MACC1-GFP cells was observed whereas the Y379F+Y789F expressing cells show proliferation on control cell level. In total, this indicates a strong influence of MACC1 phosphorylation status (Y379+Y789) and mediated signaling via the associated interactions on cancer cell migration and proliferation.

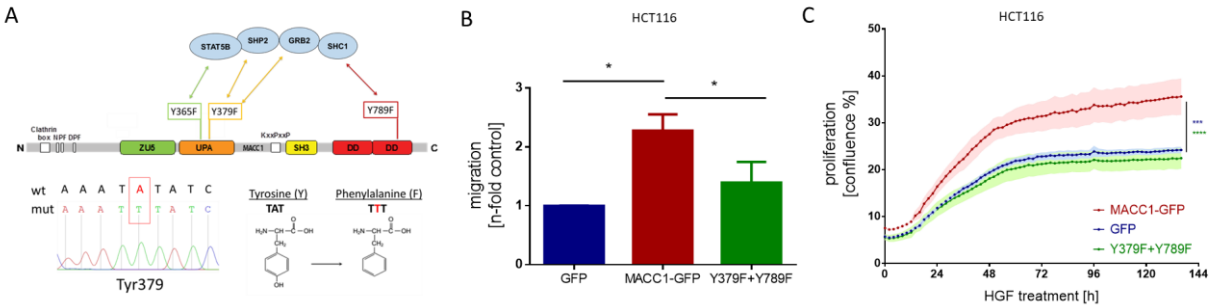


Fig. 23: pY-interaction sites are important for MACC1 function. (A) Schematic domain architecture of MACC1 including identified pY-interaction sites and binding partners. Further, a representative example of site-directed mutagenesis performed at Y379. The single base substitution from adenosine to thymidine leads to an amino acid switch from tyrosine to the structural similar phenylalanine which is not accessible for phosphorylation. **(B)** HCT116/MACC1-GFP cells demonstrate significantly elevated levels of cell migration in the Boyden chamber assay compared to control cells. In contrast, mutation of MACC1-GFP at Y379 and Y789 (Y379F+Y789F) reduced migratory potential in HCT116 cells expressing this construct almost to control level. **(C)** Similarly, HGF-induced proliferation assessed by IncuCyte® live imaging was increased through MACC1-GFP overexpression whereas mutation at Y379 and Y789 (Y379F+Y789F) led to proliferation on control cell level. Results represent means + SEM of three independent experiments. Significant results were determined by one-way (Fig. 23B) or two-way (Fig. 23C) ANOVA and Turkey´s or Sidak´s multiple comparison test with a confidence interval of 95% (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$). Blue - GFP expressing cells; red - MACC1-GFP expressing cells; green - MACC1-GFP (Y379F+Y789F) expressing cells.

3.2.3 MACC1 facilitates downstream signaling of receptor tyrosine kinases

MACC1 was shown to exert some of its functions such as induction of proliferation and cell scattering in response to HGF stimulation [100]. In this study, we identified and confirmed interactors of MACC1 which play important roles in signal transduction of RTKs such as MET [23,42,44-56]. Therefore, we aimed to investigate the effect of MACC1 on receptor tyrosine kinase signaling by performing stimulation experiments with HGF plus subsequent WB of the downstream effector ERK and activating phosphorylation at T202 and Y204. Concomitantly, MACC1 expression was determined. Vinculin served as loading control. The WB pictures show one representative example whereas bar graphs represent the normalized signal strength of three independent experiments analyzed by spot densitometry which correlates to the respective protein expression levels. Here, expression of pERK and ERK were

normalized to vinculin expression before normalizing pERK to ERK indicating the level of active protein. Values for untreated (0 min) HCT116/GFP or SW480/ev cells were set to 1 resulting in relative expression levels depicted in the respective bar graphs.

Figure 24 A and B show an increase of ERK phosphorylation upon growth factor treatment indicating the activation of the MET signal transduction cascade. Interestingly, the activating phosphorylation of ERK demonstrate a stronger amplification in the cell lines overexpressing MACC1 compared to the respective empty vector cell lines over the time period of stimulation, especially at 1 and 8 min. pERK levels are also higher in MACC1 overexpressing cells at the unstimulated start point of the experiment (0 min). After 90 min, ERK activation reaches a plateau in control cells whereas MACC1 high cells demonstrate already subsiding pERK levels. This indicates an activation loop with increased velocity and signal strength through MACC1 overexpression. These findings hold true for both cell models tested.

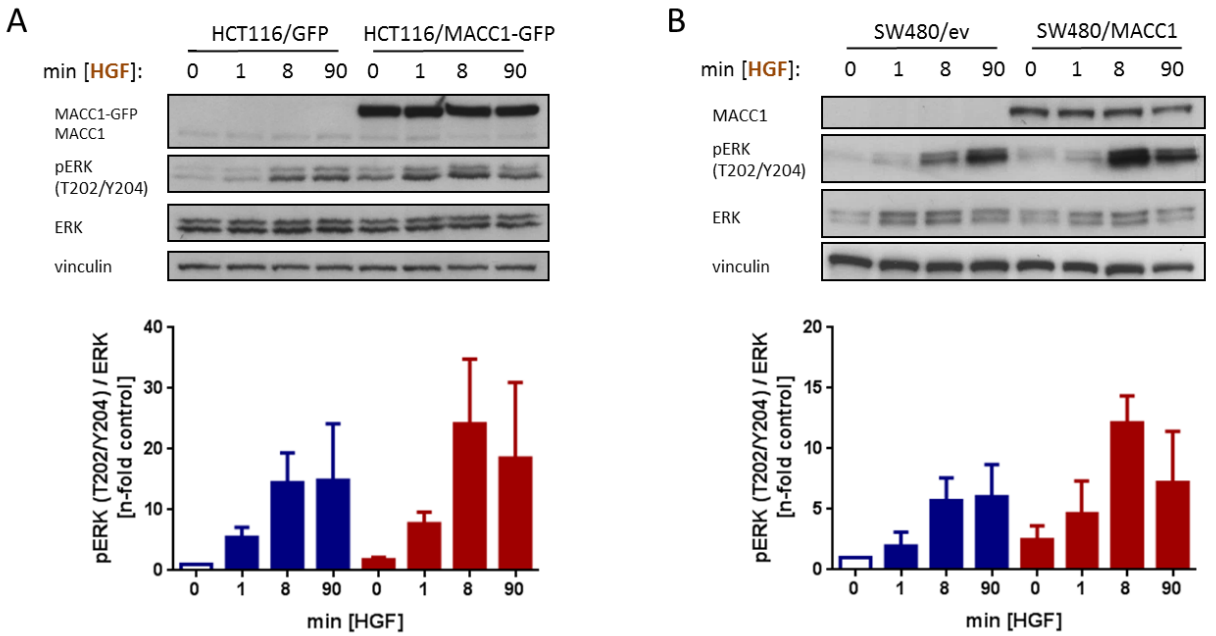


Fig. 24: MACC1 facilitates downstream signaling of MET. Cells were serum starved and, subsequently, treated with 20 ng/ml HGF for 0, 1, 8 and 90 min, respectively. As downstream target of RTK signaling the activation of ERK via phosphorylation at T202 and Y204 was determined in WB. This experiment was performed in two different CRC cell models with modulations in MACC1 expression: **(A)** HCT116/GFP vs. HCT116/MACC1-GFP and **(B)** SW480/ev vs. SW480/MACC1. Growth factor stimulation led to an increased pERK expression peaking at 8 min in the MACC1 overexpressing cell lines whereas control cell lines showed an expression plateau after 8 and 90 min. The pERK activation was augmented by MACC1 overexpression. Especially at 1 min and 8 min, pERK levels were higher in MACC1 overexpressing cells than control cells. Interestingly, the same was also seen in untreated cells (0 min). Results are demonstrated by WB and respective spot densitometry analysis. Here, expression of pERK and ERK were normalized to vinculin (loading control) expression before normalizing pERK to ERK indicating the level of active protein. Values for untreated (0 min) HCT116/GFP or SW480/ev cells were set to 1 resulting in relative expression levels depicted in the respective bar graphs. Results represent means + SEM of three independent experiments. Blue - GFP expressing cells; red - MACC1-GFP expressing cells.

The identified interactors are not only involved in signalosome formation and regulation of downstream signaling of MET, but provide similar functions for further RTKs, e.g. EGFR [323-325]. Therefore, we additionally investigated the effect of MACC1 on further receptor tyrosine kinase signaling by performing stimulation experiments with EGF. Similar to figure 24, WB was used to determine the activation of the downstream effector ERK by activating phosphorylation at T202 and Y204. We observed according expression patterns for both cell models with pERK levels peaking after 8 min of growth factor stimulation. Also upon EGF treatment, MACC1 overexpression applied a stronger and faster ERK activation loop to the cells (Fig. 25 A+B).

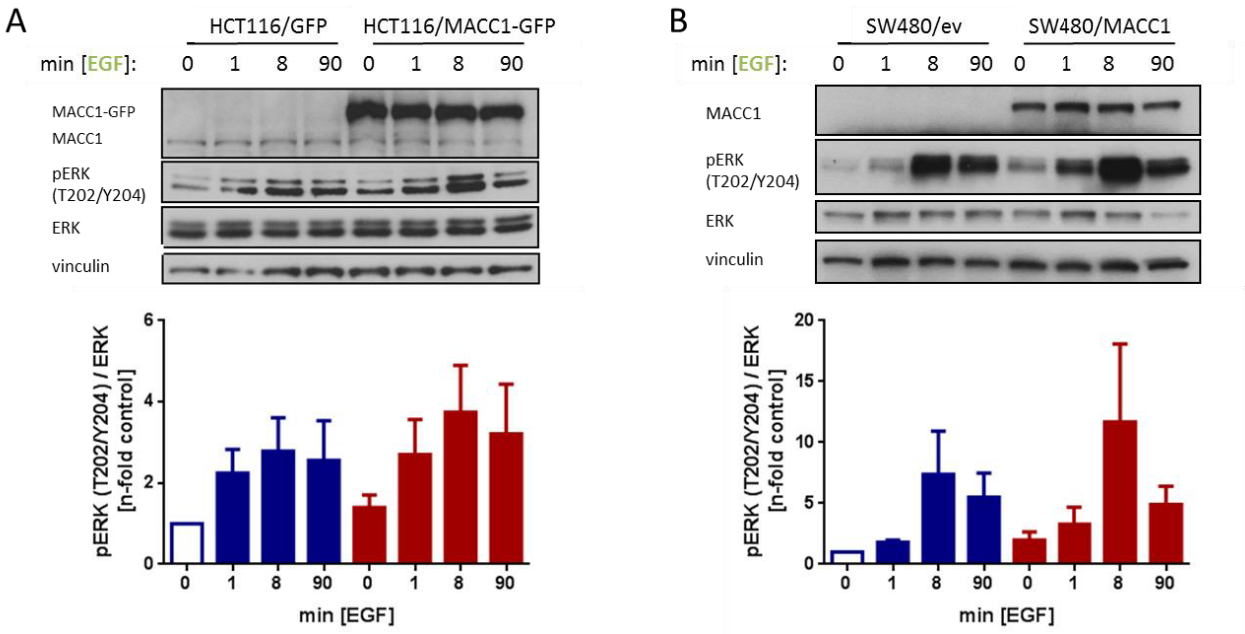


Fig. 25: MACC1 facilitates downstream signaling of EGFR. Cells were serum starved and, subsequently, treated with 20 ng/ml EGF for 0, 1, 8 and 90 min, respectively. As downstream target of RTK signaling the activation of ERK via phosphorylation at T202 and Y204 was determined in WB. This experiment was performed in two different CRC cell models with modulations in MACC1 expression: **(A)** HCT116/GFP vs. HCT116/MACC1-GFP cells and **(B)** SW480/ev vs. SW480/MACC1 cells. Growth factor stimulation led to a pERK activation loop peaking at 8 min in all four cell lines. This activation loop was augmented by MACC1 overexpression. Especially at 1 min and 8 min, pERK levels were higher in MACC1 overexpressing cells than control cells. Interestingly, the same was also seen in untreated cells (0 min). Results are demonstrated by WB and respective spot densitometry analysis. Here, expression of pERK and ERK were normalized to vinculin (loading control) expression before normalizing pERK to ERK indicating the level of active protein. Values for untreated (0 min) HCT116/GFP or SW480/ev cells were set to 1 resulting in relative expression levels depicted in the respective bar graphs. Results represent means + SEM of three independent experiments. Blue - GFP expressing cells; red - MACC1-GFP expressing cells.

3.2.4 MACC1 induced signaling is abrogated by mutation of specific tyrosine sites

As described previously, the MACC1 interactors GRB2, SHP2, SHC1 and PLCG1 are implicated in RTK signal transduction and regulation of cellular signaling like the ERK pathway thereby governing many decisive processes such as proliferation or migration [23,42,44-56].

To examine the role of the identified pY-interaction sites in MACC1-dependent RTK downstream signaling, we subjected the previously created tyrosine mutated MACC1 cell line (Y379F+Y789F) to HGF treatment and subsequent WB. Results are shown in figure 26. The bar graphs depict the results of spot densitometry analysis. Normalization was performed as described before in 3.2.3. Due to the lack of a WB signal at 0 min for HCT116/GFP cells in one of the experiments, normalization to untreated GFP control cells was not conducted and values represent arbitrary units instead of fold changes to control. The results in figure 26 also demonstrate the MACC1-dependet increase in ERK phosphorylation whereas the Y379F+Y789F cells lack this amplification and demonstrate pERK levels similar or below control after 1 min and 8 min.

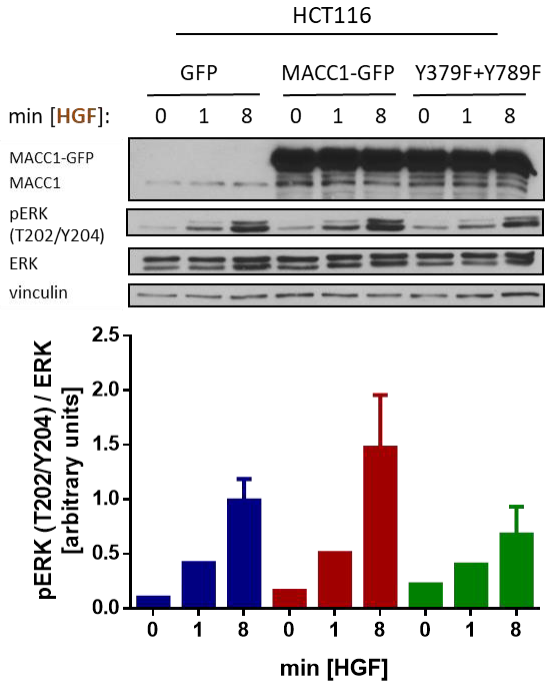


Fig. 26: The MACC1 signaling effect is abrogated by mutation of specific tyrosine sites. ERK activation (phosphorylation at T202/Y204) upon HGF stimulation is reduced in HCT116 cells expressing tyrosine mutated MACC1-GFP (Y379F+Y789F) compared to wild-type MACC1-GFP, especially after 8 min. Here, pERK levels are even lower than in GFP control cells. Expression of pERK and ERK were normalized to vinculin (loading control) expression before normalizing pERK to ERK indicating the level of active protein. Results represent means + SEM of two independent experiments. Blue - GFP expressing cells; red - MACC1-GFP expressing cells; green - MACC1-GFP (Y379F+Y789F) expressing cells.

3.2.5 MACC1 exerts a hyperactivated signaling phenotype - DigiWest

Cellular signaling pathways share high levels of crosstalk and redundancy (see 1.2.2) [23,26]. Therefore, we wanted to evaluate the effect of MACC1 on the cellular signaling landscape using DigiWest. This allowed the protein expression assessment of a large target variety from a single standard WB membrane. Proteins on the membrane were stripped and labeled via biotinylation and streptavidin-coated colored beads. Bead-pools reconstituting different membrane lanes were subjected to antibody-based immunoassays (PE-labeled secondary antibodies) with subsequent fluorescent read-out for respective protein expressions. We used the HCT116/GFP and MACC1-GFP cell pair plus HFG treatment for 0, 1, 8 and 90 min, respectively. Figures 27-30 show the protein expression of different phosphorylated proteins presented in corresponding normalization to previous experiments (3.2.3 and 3.2.4).

The relative expression of pMEK (S217/S221) (Fig. 27A), pERK1 (T202/Y204), pERK2 (T185/Y187) (Fig. 27B) and p-p90 ribosomal s6 kinase (pRSK) (T573) (Fig. 27C) indicating the activated proteins are depicted in figure 27. Again, we observed an increased phosphorylation upon HGF treatment from 0 to 8 min which subsided at 90 min. A stronger increase was seen in MACC1 overexpressing cells at 1 and 8 min accompanied by an also stronger decrease at 90 min. Untreated MACC1 overexpressing cells showed already higher pERK and pRSK levels than controls. This faster and stronger activation loop of ERK signaling corroborated previous results (3.2.3) and served as baseline for the following findings.

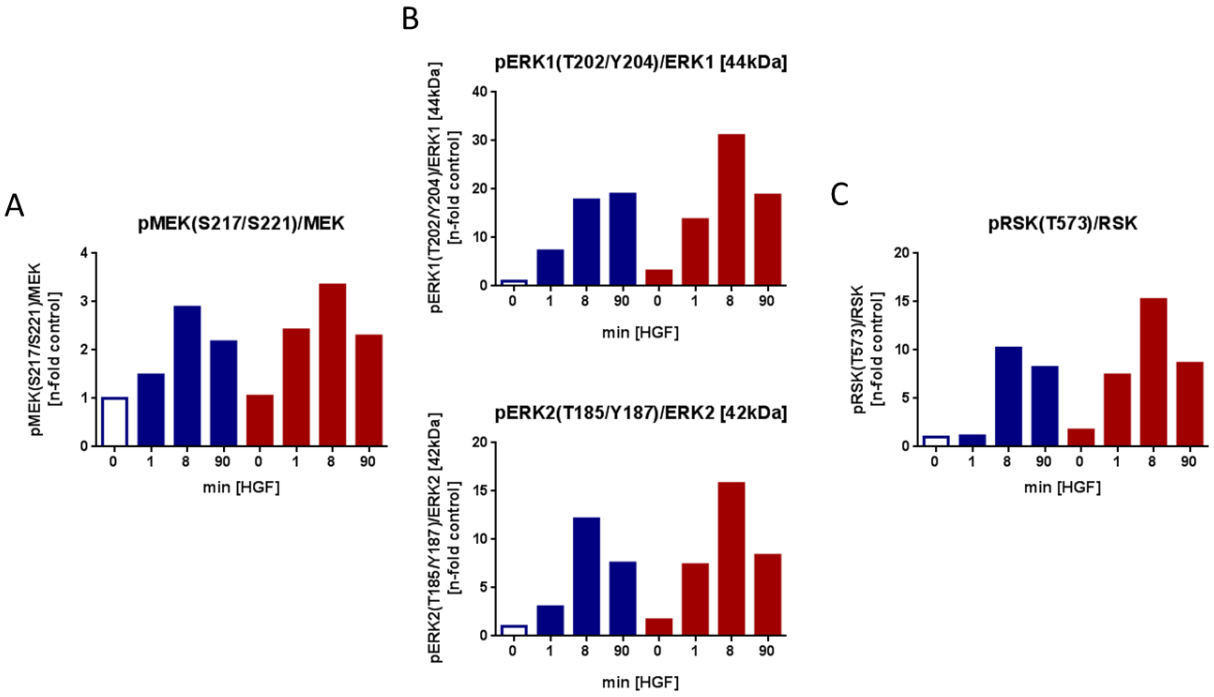


Fig. 27: MACC1 exerts hyperactivated ERK signaling. A large set of protein and phospho-protein expression in response to HGF treatment (0, 1, 8, 90 min) was assessed by DigiWest. Bar graphs illustrate integrated signal strength of phosphorylated proteins normalized to respective total protein expression indicating activated or inactivated protein levels. Stronger activation loops are found in MACC1-GFP overexpressing compared to control cells for different members of the ERK signaling pathway: **(A)** pMEK (S217/S221), **(B)** pERK1 (T202/Y204), pERK2 (T185/Y187) and **(C)** pRSK (T573). Blue - GFP expressing cells; red - MACC1-GFP expressing cells.

Similarly, expression of pGSK3β (S9; inactivated form) (Fig. 28A) and β-catenin (S552; activated form) (Fig. 28B) were comparably higher in HCT116/MACC1-GFP than HCT116/GFP cells for all time points. The cyclic activation pattern was also observed here (Fig. 28 A+B). Further, activating phosphorylation of SRC (Y416) (Fig. 29A) and focal adhesion kinase (FAK) (Y397) were strongly augmented in MACC1 overexpressing cells compared to respective controls for all time points whereas sequentially active FAK forms pFAK (Y576/577) and pFAK (Y925) showed higher expressions at 1 or 8 min, respectively (Fig. 29C). In figure 30, the results depict an increased expression for activated cAMP response element-binding protein (pCREB) (S133) in MACC1 high expressing cells from 0 to 8 min followed by a stronger drop at 90 min (Fig. 30A). The expression of active vasodilator-stimulated phosphoprotein (pVASP) (S157) was similarly higher in MACC1-GFP overexpressing cells from 0 to 8 min and lower at 90 min with a concomitant activation decline over time (Fig. 30B).

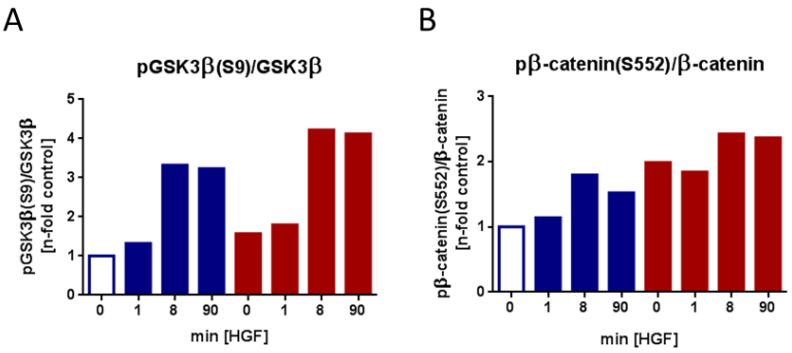


Fig. 28: MACC1 exerts hyperactivated β-catenin signaling. A large set of protein and phospho-protein expression in response to HGF treatment (0, 1, 8, 90 min) was assessed by DigiWest. Bar graphs illustrate integrated signal strength of phosphorylated proteins normalized to respective total protein expression indicating activated or inactivated protein levels. **(A)** The inactivating phosphorylation of GSK3β at S9 and **(B)** the activating phosphorylation of β-catenin at S552 show an earlier and stronger activation in HCT116/MACC1-GFP vs. HCT116/GFP cells. Blue - GFP expressing cells; red - MACC1-GFP expressing cells.

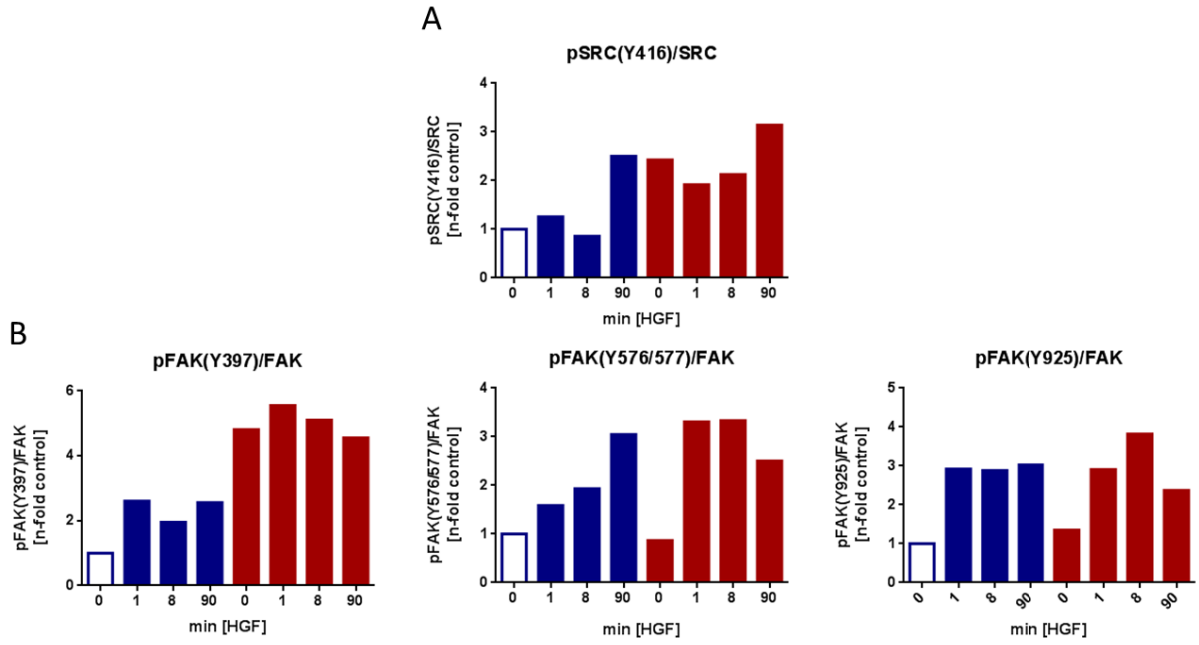


Fig. 29: MACC1 exerts hyperactivated FAK/SRC signaling. A large set of protein and phospho-protein expression in response to HGF treatment (0, 1, 8, 90 min) was assessed by DigiWest. Bar graphs illustrate integrated signal strength of phosphorylated proteins normalized to respective total protein expression indicating activated or inactivated protein levels. **(A)** Here, a drastic upregulation of SRC phosphorylation (Y416) was observed through MACC1-GFP overexpression. **(B)** The sequential, activating phosphorylations of FAK at Y379, Y576/577 and Y925 were earlier and stronger increased in HCT116/MACC1-GFP compared to HCT116/GFP cells. Blue - GFP expressing cells; red - MACC1-GFP expressing cells.

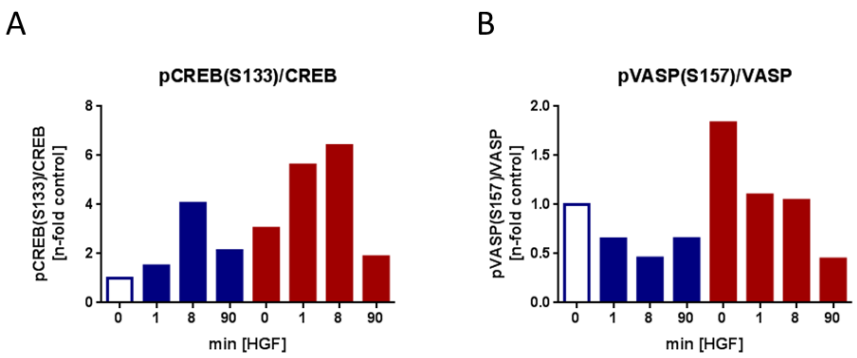


Fig. 30: MACC1 exerts hyperactivated cAMP signaling. A large set of protein and phospho-protein expression in response to HGF treatment (0, 1, 8, 90 min) was assessed by DigiWest. Bar graphs illustrate integrated signal strength of phosphorylated proteins normalized to respective total protein expression indicating activated or inactivated protein levels. The downstream effectors of cAMP signaling CREB and VASP were also affected by MACC1-GFP overexpression: **(A)** The phosphorylation of CREB at S133 demonstrated a faster and stronger activation cycle whereas **(B)** pVASP (S157) levels were basally increased and showed a decline over time. Only after 90 min they reached control cell level. Blue - GFP expressing cells; red - MACC1-GFP expressing cells.

In general, the results in figure 27-30 demonstrate an activating influence of MACC1 on several central signaling molecules involved in different cellular pathways. In most cases, MACC1 overexpression leads to faster and/or stronger activation loops with already elevated baseline levels.

3.2.6 MACC1 exerts a hyperactivated signaling phenotype - PamChip®

In an additional assessment of the signaling phenotype via the PamChip® assay, kinase activity was determined by using immobilized substrate sequences in the presence of whole cell lysate, ATP and anti-pY or anti -pS/T antibodies with fluorescent label. In this case SW480/ev and two different MACC1 overexpressing cell clones (SW480/MACC1 #10 and #43) as well as HCT116/GFP and HCT116/MACC1-GFP cells were compared. Figure 31 shows the results for the STK activity. In general, MACC1 overexpression led to an elevated STK activity indicated by increased phosphorylation of most targets compared to control cells (blue dashed line). Interestingly, augmented phosphorylation of CREB (S129/S133) and VASP (S157) were observed confirming the DigiWest findings. This was accompanied by increased phosphorylation of further members of the cAMP signaling pathway: PKA regulatory subunits alpha (S92/S99/T104) and beta (T110/S114), Phosphorylase b kinase (S1018/1020/1023) and Aurora kinase A (S283/S284/T287/T288/T292).

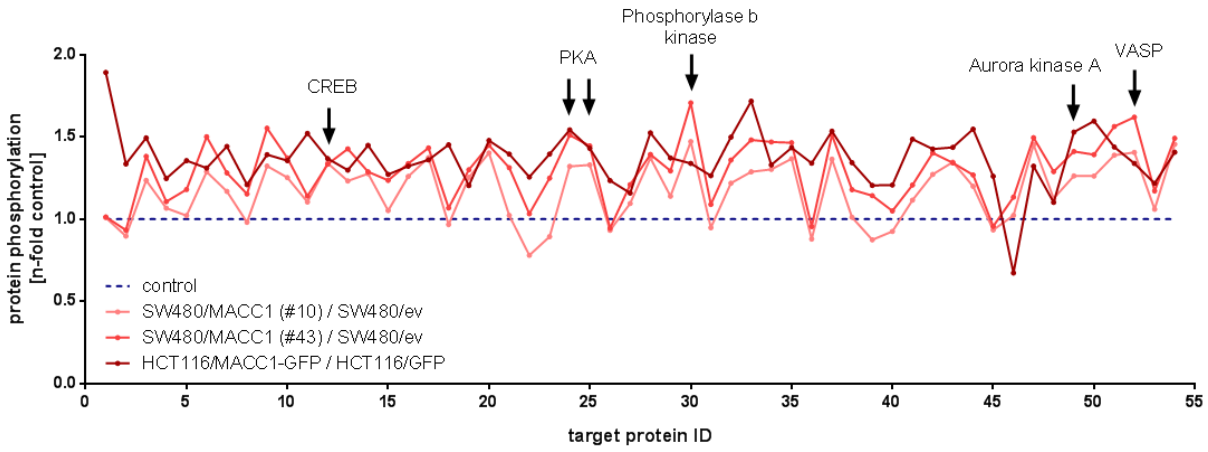


Fig. 31: MACC1 exerts a hyperactivated signaling phenotype - PamChip®. Kinase activity was determined with the PamChip® assay. Phosphorylation of several target proteins in the presence of whole cell lysates was measured with fluorescence labeled anti-pY or anti -pS/T antibodies. Results show relative phosphorylation increase of the respective target protein in MACC1 overexpressing cells (SW480/MACC1 and HCT116/MACC1-GFP, red lines) compared to control cell lines (SW480/ev and HCT116/GFP, blue dashed line). Phosphorylation of most target proteins is elevated through MACC1 overexpression in the STK assay indicating higher STK activity. A subset of stronger phosphorylated target proteins is involved in cAMP dependent signaling (black arrows): CREB, PKA, phosphorylase b kinase, Aurora kinase A and VASP.

3.2.7 MACC1 exerts a hyperactivated signaling phenotype – validation

For validation of DigiWest and PamChip® assays, we chose pSRC (Y416) and pCREB (S133) as target proteins in the HGF stimulation assay with subsequent WB. In figure 32 one representative WB and averaged spot densitometry bar graphs of two independent experiments are shown. pSRC levels are depicted in arbitrary units instead of fold changes due to the lack of a control value signal for HCT116/GFP at 0 min in one of the experiments. The results clearly illustrate that overexpression of MACC1 leads to a drastic induction of pSRC expression upon HGF treatment over time. Also, untreated cells already demonstrate higher expression levels than controls. In contrast, HCT116/GFP cells only show a brief and low pSRC expression increase with a decline already after 8 min and, further, after 90 min.

Total as well as phosphorylated CREB levels were increased by MACC1 overexpression. More importantly, the ratio of pCREB/CREB, indicating the activated protein levels, was higher in HCT116/MACC1-GFP cells for all time points, especially at 0 and 1 min. We observed a time-dependent pCREB elevation until 8 min and decline after 90 min upon HGF treatment for both cell lines. In total, these results confirm the effects of SRC and CREB activation found by DigiWest thereby validating this assay. The activation of CREB as downstream effector of PKA/cAMP signaling additionally corroborates the findings of the PamChip® assay.

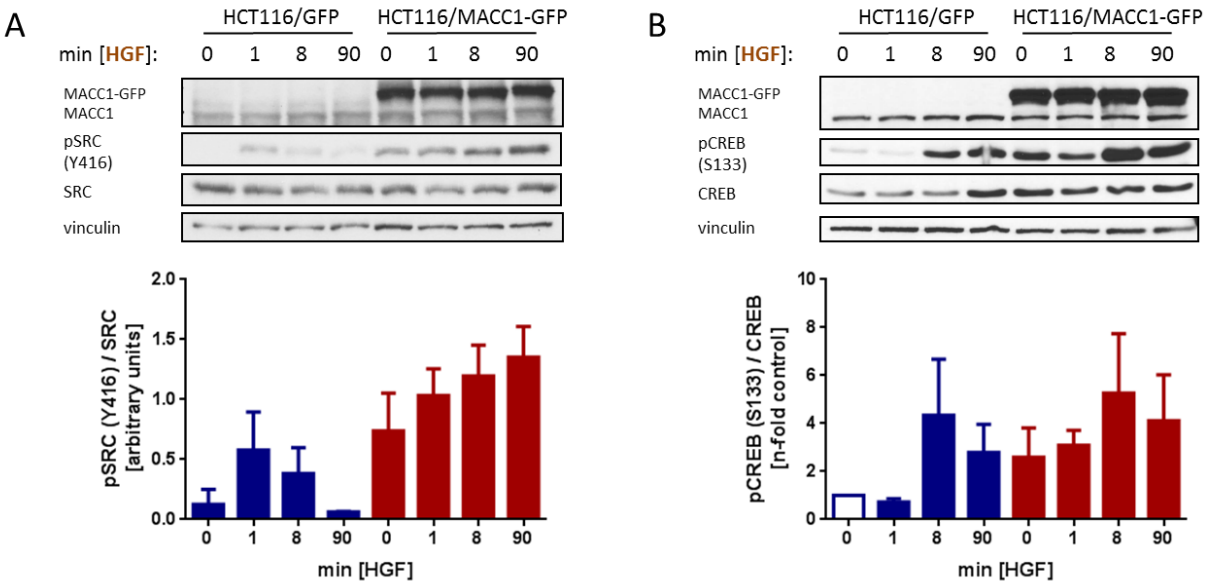


Fig. 32: MACC1 exerts a hyperactivated signaling phenotype – validation. To validate previous findings of DigiWest (3.2.5) and PamChip® (3.2.6) assay, pSRC (Y416) and pCREB (S133) levels under HGF treatment were determined with standard WB. **(A)** As seen with DigiWest, phosphorylation of SRC (Y416) is drastically increased in HCT116/MACC1-GFP compared to HCT116/GFP cells. A low and brief activation loop is observed for pSRC in GFP transduced cells whereas already high baseline levels (0 min) are further increased in MACC1-GFP overexpressing cells. **(B)** Similarly, pCREB shows higher baseline levels and stronger activation in HCT116/MACC1-GFP compare to HCT116/GFP cells. Expression of respective proteins determined with spot densitometry was normalized to vinculin (loading control) expression before normalizing pSRC to SRC and pCREB to CREB, respectively, indicating the level of active protein. Results represent means + SEM of two independent experiments. Blue - GFP expressing cells; red - MACC1-GFP expressing cells.

3.2.8 Intervention in MACC1 signaling reveals new possible treatment strategies

Currently, conventional cancer treatment strategies are increasingly supplemented or expanded with targeted intervention. Mostly, inhibitors of RTK signaling and downstream pathways are employed [162,326]. To apply this strategy to our findings, we wanted to evaluate the impact of different inhibitors on MACC1 induced signaling and function. For this, we conducted clonogenic assays in the presence of DMSO (control) or inhibitors of MET (Critzotinib, PHA-665752), SHP2 (GS493), MEK (Cobimetinib) or SRC (Dasatinib, Bosutinib, PP2). HCT116/GFP or HCT116/MACC1-GFP cells were seeded in 24-well plates at low density (200 cells/well), treated with respective drugs and colony formation was analyzed. Figure 33 shows the averaged colony covered area of at least three independent experiments with one representative example below. Values were normalized to control treated HCT116/GFP cell values (Fig. 33A; blue dashed line).

MACC1 overexpression led to a significantly increased colony formation compared to control cells (Fig. 33A). Targeted intervention of the pathways previously identified to be induced by MACC1 (3.2.3 - 3.2.7) managed to abrogate this effect. All employed inhibitors of MET (Fig.

33B), SHP2/MEK (Fig. 33C) or SRC (Fig. 33D) reduced the reproductive viability of MACC1-GFP cells to control cell level. Only Crizotinib, Cobimetinib and PP2 demonstrated minor effects on control cell colony formation.

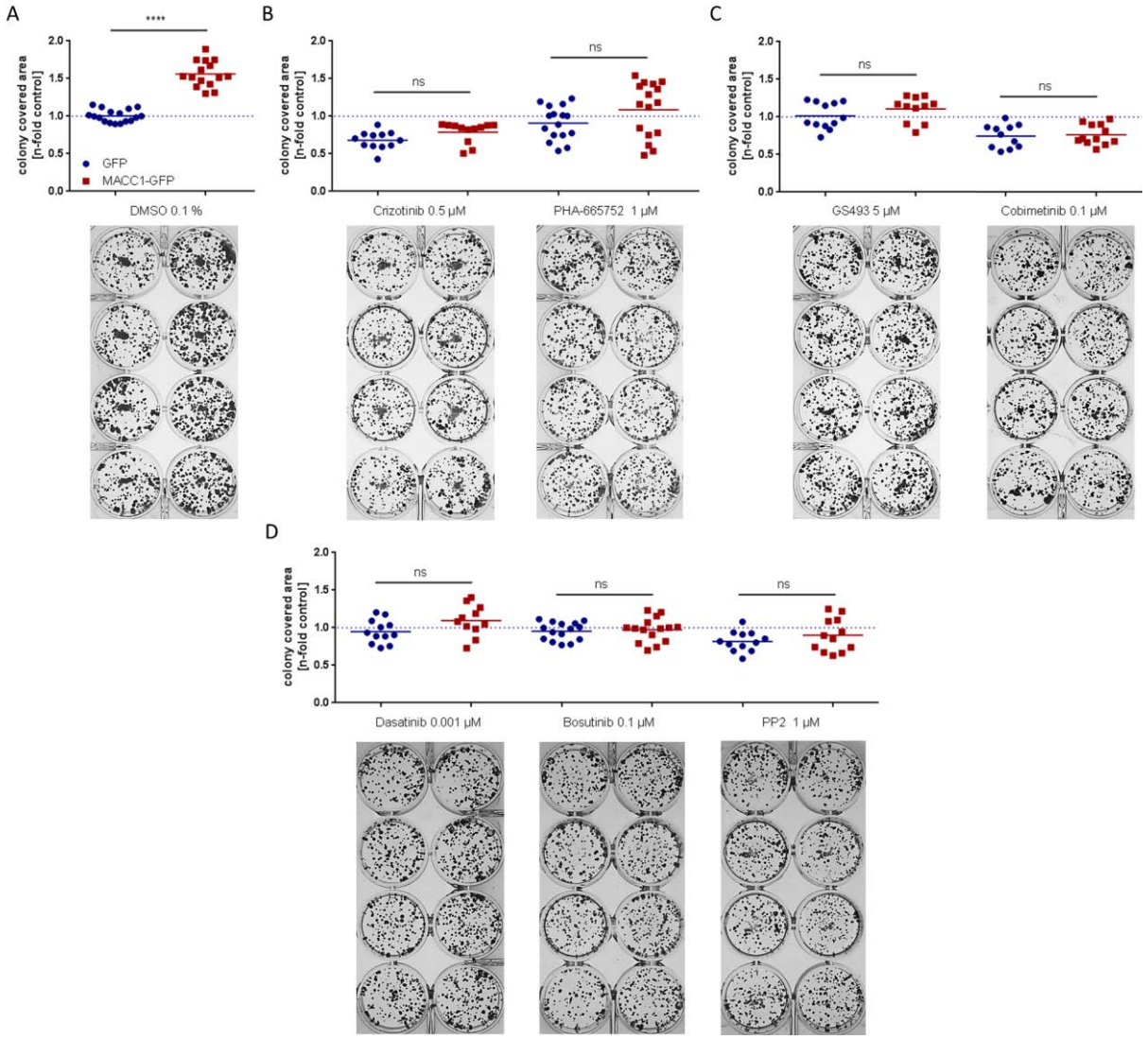


Fig. 33: Specific intervention in MACC1 signaling reveals new possible treatment strategies. Clonogenic assays in the presence of different inhibitors were performed. **(A)** Solvent (DMSO 0.1%) treated controls demonstrated a significantly augmented colony forming potential of HCT116 cells overexpressing MACC1-GFP compared to GFP control cells. **(B)** Treatment with inhibitors of MET (Crizotinib and PHA-665752), **(C)** SHP2 (GS493), MEK (Cobimetinib) or **(D)** SRC (Dasatinib, Bosutinib, PP2) reduced reproductive viability to control cell level. Colony covered area was normalized to values of solvent treated HCT116/GFP cells (blue dashed line) resulting in relative reproductive viability depicted in the dot plots. Results represent means of at least three independent experiments with one representative example illustrated below. Significant results were determined by t-test or one-way ANOVA and Turkey´s multiple comparison test with a confidence interval of 95% (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

In summary, we identified and confirmed interesting interaction partners of MACC1 via a MS-based screen and Co-IP. The interactors bind selectively to specific pY-sites on MACC1: pY365 – STAT5B; pY379 – GRB2 and SHP2; pY789 – SHC1. PLCG1 showed a more promiscuous binding pattern but favored the binding to pY379 and pY789 over their respective opposing peptide version. Mutation of two sites binding the majority of interactors (Y379F+Y789F) reduced MACC1-induced ERK activation, migration and proliferation in CRC cells. Furthermore, we demonstrated that MACC1 facilitates ERK signaling upon stimulation with HGF and EGF. By employing DigiWest and PamChip® assays, we added new layers to the MACC1 signaling landscape. Targeted inhibition of these new signaling axes restricted MACC1-promoted colony formation revealing novel intervention points for MACC1 as molecular target.

4. Discussion

Despite the intensive research of the past decades, many cancer treatment strategies present unsuccessful. Lots of chemotherapeutics show only partial effectiveness while exerting tremendous side effects due to their cytotoxic properties. Molecular targeted therapy only affects a subset of patients [143,144,166-168].

This is reflected in CRC as the second leading cause of cancer associated deaths worldwide [169]. Here, the development of distant metastasis represents a major challenge in therapy: the 5-year survival of about 10% could not be significantly improved over the last decades [106-108,170]. In addition, reliable and efficient biomarkers for early prognosis of disease course or selection of patients for specific treatment (prediction) remain scarce. The most commonly employed prognostic and/or predictive biomarkers are MMR status (MSI vs MSS) and mutations of RAS or BRAF [121,171-175]. Recently, combining of different biomarkers and molecular features resulted in consensus molecular subtypes (CMS), which should enhance patient stratification and targeted therapy [183]. This approach underlines the necessity for the establishment and application of new biomarkers for cancer treatment. One of the emerging biomarkers was identified in our group [100]: MACC1 has been established as prognostic, predictive and causal biomarker for several tumor entities [90,100,112,184-214,216-232]. So far, only two MACC1 transcriptional inhibitors have been determined [274], which highlights the necessity for additional, possibly more potent treatment strategies. Unfortunately, the revelation of the precise MACC1 protein structure remains elusive which could facilitate the generation of potential MACC1 protein inhibitors. Additionally, the MACC1 phospho-interactome as well as signaling pathways associated with MACC1 functionality have to be further elucidated.

On this basis, this study aimed to find novel, more potent transcriptional inhibitors of MACC1 and, additionally, illuminate the MACC1 signaling landscape to uncover new drug intervention points.

4.1 Transcriptional inhibition of MACC1

4.1.1 HTS identifies statins as most potent inhibitors of MACC1 expression

This study employed different strategies to identify possibilities of therapeutic intervention in high risk CRC patients by targeting MACC1 and MACC1-dependent signaling. Two distinguished screening methods built the foundation for these approaches. While the first approach demonstrated a rather classical HTS, the second one presented a newly developed method. This will be discussed in more detail later (4.2.1).

HTS developed to be one of the most widely employed methods in drug discovery. Of course, technical advances such as new or improved screening and detection systems or automated processes furthered this development [327]. In principle, an HTS is designed according to target and hypothesis. It should identify compounds from large compound libraries that affect the target in the desired manner at optimally low concentrations [328]. To identify transcriptional inhibitors, we used a luciferase reporter under control of the target (MACC1) promoter - a system already successfully demonstrated by us and others [274,329,330]. Usually, so called lead compounds are discovered which will be subjected to further drug development processes, because the HTS is not able to assess all compound properties necessary for final drug discovery: *e.g.* bioavailability, pharmacokinetics, toxicity and specificity [328]. Therefore, we chose a tiered approach: first, false positives were eliminated in a counter screen using the same cell line expressing the luciferase gene controlled by a CMV promoter. Cytotoxicity was subsequently assessed to determine the most promising compounds. They were selected based on the largest ratio between drug effect on promoter activity and cytotoxicity. This therapeutic index is a widely used measure of safety and efficacy of potential drugs [331]. Four compounds emerged, all of which were initially discovered for different applications: Fluvastatin is a member of the hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase inhibitor family used for blood lipid reduction [332]. Bosutinib and PD173952 are inhibitors of tyrosine kinases of the SRC and Abelson murine leukemia viral oncogene homolog 1 (ABL) family whereas PD161570 was found to inhibit the FGF-1 RTK [333-336]. The selected compound libraries (NIH, Microsource and Prestwick) predominantly consisted of already known and FDA approved drugs. Drug repurposing or repositioning recently advanced as strategy to find alternative applications of already approved drugs differing from their initially intended use. This approach lowers risk, time and costs of drug development mainly by avoiding the preclinical testing for drug safety [337].

In vitro validation confirmed Fluvastatin as most promising effector of MACC1 expression. Most interestingly, our previous investigation for transcriptional inhibitors in an independent

HTS using the ChemBioNet compound library revealed other members of the statin family (Mevastatin, Lovastatin) [274]. This prompted us to evaluate all clinically applied statins for their effect on MACC1 expression: The strongest effect on MACC1 mRNA expression was found for Fluvastatin (IC50: 0.85 µM) followed by Lovastatin (IC50: 1.53 µM), Atorvastatin (IC50: 1.65 µM) and Simvastatin (IC50: 3.1 µM). Pitavastatin showed more varying results indicated by larger standard errors but overall reduced MACC1 expression. These results were also demonstrated on protein level. Only here, Atorvastatin performed better than Lovastatin. Pravastatin and Rosuvastatin hardly showed any effect on MACC1 mRNA and protein expression even at high concentrations (data not shown). Interestingly, these findings correlate with the hydrophobic/hydrophilic character of the statins: Pravastatin and Rosuvastatin present a higher hydrophilicity than the remaining hydrophobic statins [338-340]. In general, all statins possess the active center with structural similarity to HMG-CoA responsible for HMG-CoA reductase (HMGCR) inhibition. Different core structures and residues attached to the active center determine the hydrophilicity and thereof metabolism as well as tissue distribution of the individual statins [341].

The metabolism of hydrophobic statins is catalyzed by cytochrome P450 enzyme (CYP) isoforms 3A4 (Atorvastatin, Lovastatin, Simvastatin) and 2C9 (Fluvastatin) whereas Pravastatin and Rosuvastatin are excreted without major CYP metabolism [342,343]. More importantly, hydrophobic statins can penetrate cell membranes by simple diffusion processes. This allows a widespread tissue distribution in comparison to hydrophilic statins which necessitate active transport via membrane transporters such as organic anion-transporting polypeptide (OATP1B1). OATP1B1 is only expressed in hepatocytes [342-344]. This would explain the differing effects of hydrophobic and hydrophilic statins on MACC1 expression *in vitro*. Concomitantly, Menter and colleagues found a variety of cancer cell lines nearly unaffected in growth by Pravastatin (hydrophilic) whereas Simvastatin (hydrophobic) strongly reduced proliferation of most cell lines. None of the cancer cell lines of entities such as colon (*i.a*. HCT116), pancreas and liver expressed OATP1B1 mRNA or protein [345]. Interestingly, the membrane transport of Pitavastatin also partly depends on OATP1B1 [346]. This might explain the varying effects of Pitavastatin on MACC1 expression. Pitavastatin also undergoes only minor CYP dependent metabolism further supporting the similarities to the hydrophilic Pravastatin and Rosuvastatin [342,343].

As described above, the originally intended use of statins is the inhibition of HMGCR which represents the central enzyme in physiological cholesterol synthesis (Fig. 34). In this pathway, HMGCR catalyzes the reaction of HMG-CoA to mevalonate, a key precursor of cholesterol [347]. The resulting decrease in intracellular cholesterol levels triggers a cascade

leading to the increased expression of low-density lipoprotein (LDL) receptors on the cell surface. Lipoproteins are responsible for the transport of triglycerides and cholesterol through the blood. LDLs shuttle from the liver to distant sites whereas high-density lipoproteins (HDL) carry the lipids from the periphery to the liver for recycling and/or excretion. By increasing LDL receptors through statin intervention, LDL uptake and degradation is facilitated leading to physiological cholesterol levels in the cell and reduced lipid levels in the blood [348].

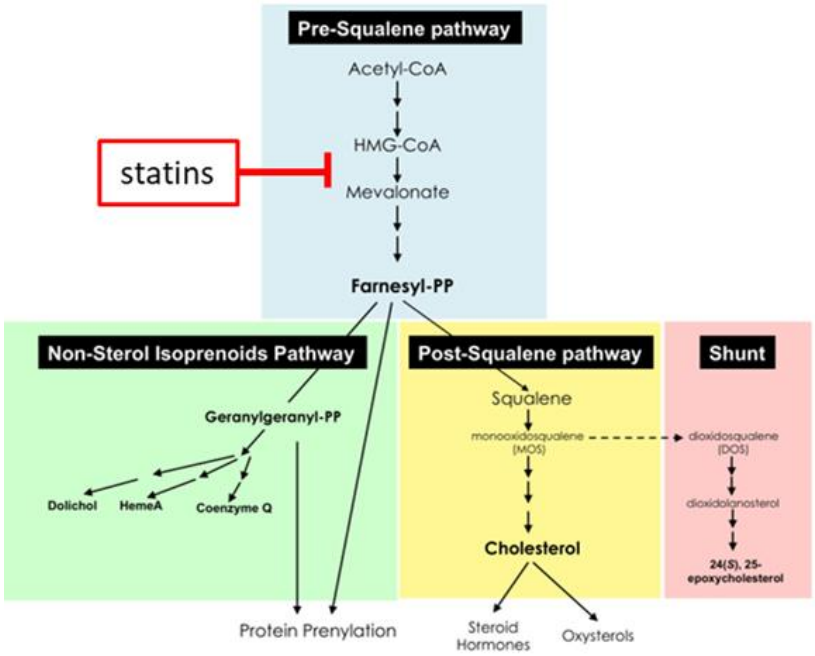


Fig. 34: The mevalonate pathway. Mevalonate is a central precursor of cholesterol, an important component of steroid hormones and the cell membrane. Other products of the mevalonate pathway, farnesyl-diphosphate (FPP) and geranyl-geranyl-diphosphate (GGPP) are used for protein prenylation. These posttranslational modifications are necessary for proper protein (membrane-) localization and function. Statins inhibit the HMG-CoA reductase (HMGCR) to block mevalonate and subsequent cholesterol production. The resulting decrease in intracellular cholesterol levels triggers a cascade leading to increased cholesterol uptake and degradation in the cells [347-353].

Since the first approval in 1987, statins have been frequently employed in prevention and therapy of cardiovascular diseases [317,354,355]. Recently, several alternative applications of statins outside the scope of their blood lipid lowering properties have emerged. Statins have been found to modulate immune response, anti-inflammatory processes and signaling pathways which involve or depend on cholesterol precursors. Therefore, statin use is now connected to a variety of diseases such as multiple sclerosis, inflammatory bowel diseases, rheumatoid arthritis, systemic lupus erythematosus, HIV and cancer [348,356,357]. Several studies already reported the inhibiting effects of statins on tumor cell expansion *in vitro* and *in vivo* [358-368].

In accordance with this, we demonstrated in this study that statins reduce MACC1-dependent, malignant, cellular functions such as proliferation or colony formation and restrict tumor growth as well as metastasis formation in a xenografted mouse model for CRC. Previously, we already documented the effect of Lovastatin on MACC1-dependent migration and wound healing *in vitro* as well as tumor growth and metastasis formation *in vivo* [274]. With this study, we expanded the statin effect on MACC1 expression to nearly all members of the statin family and to further MACC1-dependent cellular functions. Most importantly, we demonstrated restricted tumor growth and metastasis formation with daily doses of 13 mg/kg body weight (compared to 100 mg/kg body weight [274]) which represents a human equivalent dose of approximately 1 mg/kg body weight [316]. This dose is commonly employed in blood lipid reduction therapy [317].

4.1.2 Statins specifically inhibit MACC1-dependent functions *in vitro* and *in vivo*

Regarding the mode of action, we previously reported that statins hinder the binding of transcription factors AP-1 and SP1 to the MACC1 promoter leading to diminished transcriptional activation and MACC1 expression. *In silico* molecular docking analysis additionally showed that statins can competitively bind to the DNA binding pocket (leucine zipper) of AP-1 providing more insight into the mechanism of statins acting on MACC1 expression. To proof the specificity of the inhibiting effect of statins on MACC1-dependent cellular functions, we ectopically overexpressed MACC1 under control of a different promoter (CMV promoter) which rescued *in vitro* migration and wound healing [274]. In this study, we corroborated these findings for MACC1-dependent proliferation and clonogenicity with the overexpression of either MACC1-GFP under control of a CMV promoter or MACC1 under control of a tetracycline responsive inducible promoter. Additionally, MACC1 knock-out led to strongly reduced proliferation which was not further reduced by statin treatment. *In vivo*, we found decreased amounts of MACC1 transcripts in the liver metastasis of statin treated xenografted mice in this as well as the previous study. Also, the mRNA expression of several other proposed transcriptional target genes of statins were not significantly modulated by statin treatment *in vivo*. Only Col1A1 expression was significantly reduced whereas HMGCR showed strikingly augmented mRNA expression [274]. Statin-induced HMGCR feedback induction has already been demonstrated by others as well [369]. Nevertheless, MACC1 expression appears unaffected by HMGCR upregulation *in vitro* and *in vivo* in this and the previous study [274].

None of the observed effects was absolute and transcriptional inhibition of MACC1 cannot be claimed as the only mechanism of statins perturbing tumor development. This notion was

further supported by the finding that MACC1 expression in HCT116 cells was partly restored by replenishing culture medium with mevalonate (data not shown). Moon and colleagues further suggested the dependency of mevalonate pathway activity and subsequent vulnerability to statin treatment on P53 status in HCC [366]. This is also reflected in the data of this and our previous study [274]: The CRC cell lines with P53 wt (HCT116, SW48) show reduction of MACC1 expression at lower concentrations than these with mutated P53 (HT29, DLD1, SW620) indicating the potentially higher mevalonate pathway activity in these cell lines [366,370-372]. In an oncogene dependent context, the mevalonate pathway was shown to induce ERK signaling druggable with statins [373]. Thus, intervening in the mevalonate pathway interferes in the proposed MACC1/MET/ERK/MACC1 positive feed-back loop providing a potential mechanism for the effect of statins on MACC1 expression independent of transcriptional inhibition [215].

The mevalonate pathway further provides crucial end-products such as farnesyl-diphosphate (FPP) and geranyl-geranyl-diphosphate (GGPP) important for protein isoprenylation. These posttranslational modifications are necessary for proper protein (membrane-) localization and function [349-352]. Central prenylation targets are members of the RAS and RHO GTPase families involved in cancer cell proliferation and invasion [374-378]. Many studies showed decreased proliferation and invasiveness of tumor cells from different entities by targeting protein prenylation with statins [365,379-389]. We found possible interactions of MACC1 and different RHO GTPases such as RHO A, RAC1 and CDC42 by MS (unpublished data). These RHO GTPases can form complexes modulating cellular polarity thereby aggravating cancer cell migration and invasion at the leading edge of the tumor [390,391]. MACC1 was also shown to accumulate at the tumor front and tumor budding cells [230]. This indicates a potential association of these proteins to drive tumor invasion at the invasive front. Furthermore, *in silico* prediction of the MACC1 protein structure demonstrated a suggested farnesylation site providing an additional possible target for statin interference with MACC1 function (unpublished data).

The main effect conferred by statins on the molecular level represents cell cycle arrest: Statins have been found to regulate cell cycle on several levels via cyclin A, D1, E, Cyclin-dependent kinases (CDK) 2 and 4, p21, p27, P53, Checkpoint kinase (CHK) 1 and cell division cycle (CDC) 25 phosphatase in different tumor entities [360,367,386,392-396]. Recently, Wang *et al.* demonstrated restricted HCC growth through Simvastatin *in vitro* and *in vivo*. Here, statin treatment reduced phosphorylation of STAT3 leading to a downstream cascade which resulted in cell cycle arrest [358]. Another study suggested that statin treatment interfered with cell membrane cholesterol composition with subsequent ligand-

independent FAS activation and apoptosis [397]. Interestingly, our group showed that MACC1 knock-down also reduced phosphorylation of STAT1 and STAT3 leading to increased sensitivity of tumor cells to death receptor (*i.a.* FAS)-mediated apoptosis [268]. Beckwitt and colleagues further suggested the involvement of AKT signaling in statin-mediated restriction of cancer cell growth [398]. MACC1 has already been demonstrated to govern cell cycle progression through PI3K/AKT signaling and expression of several target genes, such as cyclin B, D1, D2, E, c-MYC, and SPON2 whereas MACC1 silencing - independent of interference in the mevalonate pathway - led to cell cycle arrest [221,236,237,250,251,267,271,272]. This underlines the specificity of the statin effect on MACC1 and mediated functions *in vitro* and *in vivo* presented in this study.

In total, we can observe complex and multilayered mechanisms of statins intervening in tumor growth and development. The disturbance of the mevalonate pathway and subsequent molecular consequences represents a central process, but the transcriptional inhibition of MACC1 is a non-neglectable parallel mode of action. There are also different possible intersections or mergers of both functional interventions as described in this chapter. However, the striking potential of repurposing statins for therapy or prevention of cancer (chemoprevention [399]) is obvious and will be discussed in the following chapter.

4.1.3 Statins for cancer therapy and prevention in the clinics

Chemoprevention was firstly introduced by Sporn and colleagues in 1976, when they investigated vitamin A and synthetic analogs for their cancer preventive potential [400]. It describes the continuous application of pharmacologically active substances to inhibit, delay or reverse the onset of cancer. Several agents of different categories haven already been employed, researched or discussed such as hormonal agents (*e.g.* tamoxifen), dietary agents (*e.g*. different vitamins or calcium), vaccines (*e.g.* hepatitis B virus and human papilloma virus) or pharmaceuticals (*e.g.* aspirin or metformin) [399,401].

The use of statins as chemopreventive agent is controversially discussed in the scientific literature: Some meta-analyses report the risk reduction potential of statins for different entities [402-405] whereas others observed no such effects [406-409]. Numerous further studies provide evidence pro chemopreventive effects of statins: Poynter and colleagues found a 47% reduced risk for colorectal cancer with statin use while Voorneveld *et al.* observed a 34% risk reduction [410,411]. Others showed weaker effects for CRC: HR 0.71 and HR 0.84, respectively [412,413]. The risk for pancreatic cancer was found to be lowered through statins by 39%, 34% or not significantly (OR 0.93) [414-416]. Statin effect on gastric cancer risk presented contradictory with RR 0.56, HR 0.83 and RR 1.37 [417-419].

Interestingly, statin use decreased the risk of bone metastasis of breast cancer patients (OR 0.49) and was linked to better overall survival of patients with metastatic pancreatic cancer [420,421]. We support these observations with the findings of this study that statins abate MACC1 expression in CRC, pancreatic and gastric cancer cells and specifically inhibit MACC1-mediated functions *in vitro*. More importantly, we demonstrated that statins hinder tumor growth and metastasis formation in a xenografted mouse model for CRC metastasis at concentrations equal to those used for standard statin therapy in humans [316,317]. In cooperation with the Preissner group at the Charité, we further applied a real-world evidence (RWE) study design to a large transatlantic cohort of 53,113 cancer patients. Here, statins presented an overall cancer risk reduction of 50%. We additionally identified strong risk reductions for individual cancer entities and metastasis by different statins (Gohlke & Zincke *et al*., submitted).

Obviously, the results of clinical trials strongly depend on study design including cohort population and randomization as well as vulnerability to certain bias [422]. Modern clinical trials certainly try to decrease the influence of bias and follow precise protocols to ascertain proper data generation and evaluation. By application of these strict criteria, especially for patient enrollment, they partially disregard the real-world situation with higher variability in patient age, disease severity or comedications and comorbidities. Moreover, present expanses for clinical trials are high, with predictions of increase, and they consume a lot of time and resources. To account for this, generation of RWE has gained increasing interest. It makes use of the expanding electronic health records and administrative data which consistently monitor and reflect patient health status and care [423,424].

Because of the usually long latency of a treatment effect and the predominant fatality of cancer diagnosis, the discovery and employment of biomarkers is inevitable for chemoprevention [399]. They help identifying the population at high risk for a certain disease course (prognostic biomarker) or can predict responsiveness to treatment (predictive biomarker) [425]. In view of the current literature and our present data, we recommend chemoprevention with statins in patients stratified for MACC1 expression. This is especially sensible because MACC1 serves as direct target of statins. For the evaluation of the predictive character of MACC1 upon statin treatment, more clinical research is warranted. Here, a combined approach with P53 status might be of interest as Moon and colleagues independently suggested P53 status as prognostic and predictive marker for statin therapy [366].

4.2 MACC1 phospho-interactome and signaling landscape

4.2.1 MACC1 interacts with crucial proteins promoting a malignant phenotype

The second part of this study intended to uncover more details of the MACC1 signaling landscape, thereby revealing potential drug intervention points. As previously indicated, it was based on an MS screen to identify interactions of tyrosine-phosphorylated MACC1. Tyrosine phosphorylation represents a crucial mechanistic switch in signal transduction. Phosphorylation of specific Y-sites mostly leads to an activation of the respective protein. Phosphorylated tyrosines (pY) additionally serve as docking sites for SH2 domain containing proteins, thereby fostering downstream signaling [296-299]. Therefore, we aimed to identify pY-dependent interactions of MACC1 in CRC. The MS screen was originally set up and employed for kinase inhibitor profiling: It used sepharose-immobilized kinase inhibitors (kinobeads) to pull down kinases from kinase inhibitor treated or untreated cell lysates to determine binding inhibition and competition [313,426,427]. Here, we applied tyrosine-phosphorylated peptides, representing all potential pY-sites on MACC1, as affinity tools to pull down interactors from whole cell lysates of SW480 and SW620 CRC cells as well as human placenta tissue. Placenta tissue is an easily accessible human primary tissue expressing a large variety of proteins: 69% of all human proteins were found to be expressed in placenta tissue compared to a 44% expression baseline detected in most other tissues [319-321]. Therefore, placenta served as control tissue.

For further analysis, we focused on interactors identified in both screens and all tissues. Here, five interactors known to be involved in RTK signalosome formation and downstream signaling particularly piqued our interest: GRB2, SHP2, SHC1, STAT5B and PLCG1 [42,44-56]. In order to biochemically validate the findings of the MS screen, we selected Co-IP experiments as one of the established standard methods for identification and verification of protein-protein interaction [322]. All five interactions were confirmed in SW480/MACC1 and SW620 cells.

The initial screen already indicated the binding of the interactors to specific pY-sites on MACC1. With further pull-down experiments using the respective phosphorylated and unphosphorylated pY-peptides, we showed the selective binding of SHP2 and GRB2 to the phosphorylated Y379 peptide whereas SHC1 and STAT5B interacted with pY789 and pY365, respectively. PLCG1 binding was rather unspecific: It showed the strongest interaction with pY379 followed by pY789 and contradicting results for Y365. This prompted us to perform SDM at the respective pY-sites to evaluate their functional potential. First, we focused on the interaction sites Y379 and Y789 which bind the majority of interactors: GRB2, SHP2, SHC1, PLCG1. All of these have been found to regulate cellular signaling like the

ERK pathway governing many decisive processes such as proliferation or migration [23,42,44-56].

Indeed, we found impaired migration and proliferation in CRC cells expressing MACC1-GFP carrying mutations at Y379 and Y789 (Y379F+Y789F) compared to those expressing wild-type MACC1-GFP. They showed migration close to and proliferation on control cell level. Moreover, we demonstrated that HGF-induced ERK activation was reduced upon mutation of these sites. This indicates a strong influence of MACC1 phosphorylation status (Y379+Y789) and mediated signaling via the associated interactions on these cellular processes in CRC. This is a phenomenon already observed in different studies. For instance, phosphorylation and subsequent SHP2 binding of Y627 and Y659 on GAB1 has been shown to be crucial for downstream activation of the ERK pathway in response to different stimuli such as HGF, EGF, VEGF, Lysophosphatidic acid (LPA) or interleukin-6 (IL-6). GAB1 with mutations at these sites failed to mediate corresponding biological functions *in vitro* and *in vivo* including cell migration, cytoskeletal reorganization or organ development [52,53,428-435]. Bertotti and colleagues demonstrated the importance of (by MET) phosphorylated tyrosine sites Y1257, Y1440 and Y1494 on β4-integrin for SHP2 binding and downstream signaling. Mutation of these sites prevented SHP2 binding thereby reducing anchorage-independent growth of breast cancer cells via a SHP2-SRC-GAB1-GRB2-ERK axis [436].

Preliminary Co-IP data further showed that the binding of all four interactors to MACC1 is abrogated upon mutation of both pY-sites, but more work is needed to finally validate these findings. Based on our current results, the most probable route of interaction occurs between the pY-site on MACC1 and the SH2 domain occurring in all interactors. SH2 domains present approximately 100 amino acid long protein elements that specifically recognize and bind pY-sites. The surrounding amino acids of the pY-site ranging from -2 to +4 (in some cases even -6 to +6) position play a crucial role in the selectivity for the target protein [297,437,438].

For instance, the SH2 domain of SHC1 preferentially binds pYXXΦ motifs, where X represents a random and Φ a hydrophobic amino acid, which fits to the MACC1 sequence at Y789 (pYDFL) [439]. GRB2 characteristically interacts with motifs such as pY(L/V)N(V/P) [438,440]. The corresponding sequence on MACC1 at Y379 (pYIHP) shows close similarities: While the proline (P) already fits the motif, valine (V) or leucine (L) could be substituted by their close relative isoleucine (I) since they only differ in occurrence or position of one methyl group, respectively. GRB2 is also able to bind the pYVNV motif on SHC1 providing the additional possibility of indirect interaction with MACC1 [441]. STAT5 has been shown to predominantly interact with motifs harboring an acidic amino acid (*e.g.* aspartic acid, D) in -1 and aliphatic amino acids such as leucine, isoleucine or valine at +1 and +3 position of the pY which also presents a strong overlap with the suggested Y365 (DpYIHK)

binding site on MACC1 [442]. SHP2 prefers binding to so called immuno-receptor tyrosine-based inhibitory motifs (ITIMs): (I/V/L)XpYXX(I/V/L) [443]. While this motif is not specifically found at Y379 on MACC1, newer findings suggest a binding dependence on aliphatic amino acids at +1 and +3 position and a significant involvement of large hydrophobic or positively charged amino acids at +4 and/or +5 position [444,445]. This, in contrast, occurs on MACC1 with an isoleucine at +1 and a phenylalanine (F) at +5 position of Y379 (pYIHPSF) supporting the proposed SH2-dependent interaction. Known and potential binding motifs of proteins interacting with PLCG1 SH2 domains comprise following sequence: pY(I/V/L)X(I/L/V/P) [438]. This also clearly resembles the Y379 site of MACC1. PLCG1 has been found to interact with a wide variety of proteins and additionally binds phospholipids via its SH2 domains [446-448]. Therefore, motif selectivity and specificity might present rather promiscuous which supports our pull-down data.

In general, none of these motifs provide absolute assurance of specific interaction considering the small protein-protein interface. Best physiological binding is also not inevitably achieved by the most precise interaction motif. The recurring use of small domains and motifs however promoted the development and complexity of cellular functions by enhancing protein networking independent of gross changes in proteins or gene number [449]. On the other hand, this high level of flexibility and redundancy is one of the major issues in cancer and cancer therapy, respectively (see 1.2.2 and 1.2.4).

Besides the SH2 domain dependent binding of MACC1 pY-sites, there are additional potential modes of interaction (Fig. 35): SHC1 contains a phosphotyrosine binding (PTB) domain with similar function as the SH2 domain. It preferentially binds to NPXpY motifs, which also displays similarities with the Y789 (KPApY) site on MACC1. Additionally, it harbors a proline-rich domain which can interact with the SH3 domain in MACC1 [439,450-452]. PLCG1 and GRB2 respectively possess one and two SH3 domains able to bind to the proline-rich domain of MACC1 [453,454]. SHP2 provides an additional catalytic protein tyrosine phosphatase (PTP) domain for the execution of its protein dephosphorylating functions. This demonstrates substrate specificity for motifs containing one or more acidic (and aromatic hydrophobic) amino acids on both sides of the pY-site [455]. While these conditions are not met by the Y379 site (IYGPKpYIHPSF), Y365 (ATIWDpYIHKTT) or Y789 (MWKPApYDFLYT) might serve as substrate for SHP2. Catalytic activity usually necessitates the release from the autoinhibitory intramolecular interaction between the PTP and the N-terminal SH2 domain [456-458]. Therefore, a SH2 domain dependent binding to Y379 with subsequent dephosphorylation of Y365 or Y789 with possible negative regulation of MACC1 activity presents a possible model. SHP2, however, is predominantly associated

with the activation of several signaling pathways and cellular processes [455,456,459]. This will be discussed in more detail later.

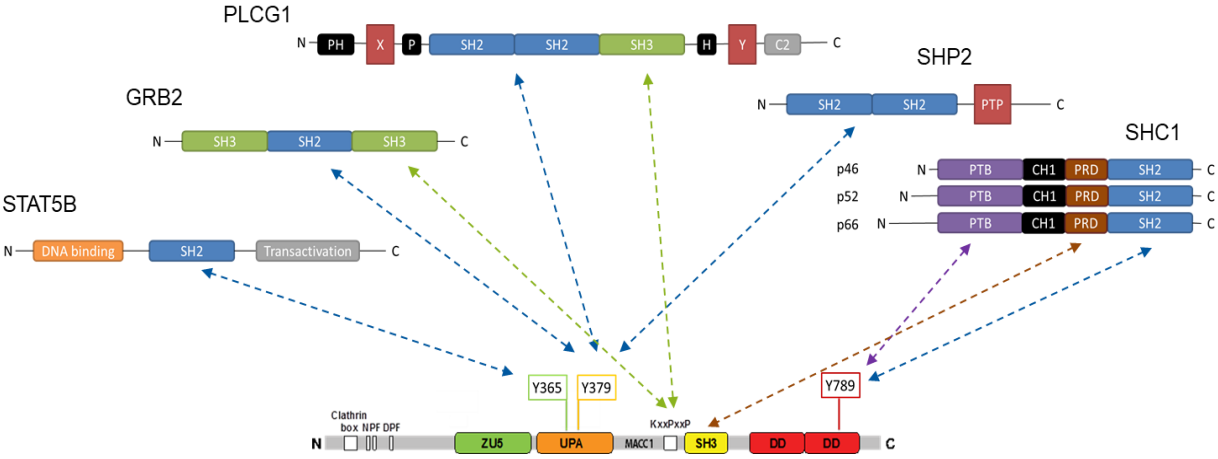


Fig. 35: Possible MACC1 domain interactions. Domain architecture of MACC1 and interactors provide several possibilities of interaction. The current results and binding motif sequences suggest binding of SH2 domains to distinct pY-sites on MACC1: SHP2, GRB2 and PLCG1 – pY379; SHC1 – pY789; STAT5B – pY365. Further binding opportunities exist between respective SH3 domains and proline rich sequences as well as the phosphotyrosine binding (PTB) domain and a pY-site. ZU5 - zonula occludens 1 and uncoordinated protein 5 resembling domain; uncoordinated protein 5 (Unc5), p53-induced death domain protein 1 and ankyrins resembling domain; SH2 - Src homology 2 domain; SH3 - Src homology 3 domain; DD - death domain; PH - Pleckstrin homology domain; PRD - proline rich domain; PTP - protein tyrosine phosphatase domain; C2 – C2 domain; X and Y – catalytic domains; CH1 – CH1 domain

In addition to the example of SHP2, there is a multitude of possibilities how different protein domains can cooperate or interfere with each other: Another negative regulation is observed in the competition of adjacent binding motifs. For instance, the chromo domain of heterochromatin protein-1 (HP1) binds the trimethylation of K9 on histone H3, which quenches gene expression. To reactivate this process in cell division, Aurora B kinase phosphorylates the juxtaposed S10 of histone H3 leading to the loss of interaction with HP1 [460,461]. To increase binding affinity and specificity, neighboring domains can attach to parallel motifs such as the SH2 domains of ζ-chain (T-cell receptor)-associated protein kinase 70 kDa (ZAP-70). One domain can also recognize multiple motifs like cell division cycle-4 (CDC4) only interacts with inhibitor of cyclin-dependent protein kinase-1 (SIC1) after phosphorylation of at least six S/T residues [462-464]. Cooperative domain interactions can also occur in a sequential pattern: Recruitment of the casitas B-lineage lymphoma proto-oncogene (CBL) to phosphorylated RTKs via its SH2 domain leads to ubiquitination of adjacent K residues providing docking sites for proteins involved in subsequent receptor endocytosis [465]. One of the most famous examples is the sequential formation of signaling

complexes at RTKs usually mediated by the central (multi)adaptor proteins GRB2, SHC1 and GAB1 [42,48,466].
In total, all these possible module and motif interactions, let alone the fact that five interactors potentially compete for three pY-sites, warrants further investigation in the spatiotemporal regulation of MACC1 signalosome formation (see 4.3).

All MACC1 interactors have been found to be involved in crucial cellular processes. SHP2 associates with GAB1 to mediate several functions including cell migration, cytoskeletal reorganization or organ development mainly via the activation of RAS/ERK signaling [52,53,428-435]. A few studies link SHP2 mutations to the development of different leukemias or cancers of colon, breast and skin [467-473]. SHP2 has also been found to promote growth and progression of lung and breast cancer as well as glioblastoma *in vitro* or *in vivo* [54,436,474,475].
GRB2 is a key adaptor protein linking RTKs to their downstream effector proteins. In most scenarios, it is continuously bound to SOS, a GEF catalyzing the RAS activation reaction, via its SH3 domain. It promotes signal transduction by linking SOS and RAS to the activated RTK (*e.g.* EGFR, MET, PDGFR) via its SH2 domain [454,476-478]. By recruiting GAB1 to RTKs, GRB2 can also mediate further signaling responses through PI3K, PLCG1 or STATs [42,47,48,52,466]. Increased GRB2 expression or function have been linked to tumor progression and metastasis formation in different entities such as breast, bladder and colorectal cancer [479-487].
In a similar manner, SHC1 mainly functions as adaptor between RTKs and the GRB2-SOS or GRB2-GAB1 complex, thereby modulating downstream signaling [466,488,489]. Consequently, SHC1 has also been reported to be implicated in oncogenic events, especially malignant cell transformation and metastasis, *e.g.* in breast or colorectal cancer [485,489-493].
Upon receptor stimulation, PLCG1 is recruited directly or indirectly via GAB1 to the receptor and its substrate phosphatidylinositol(4,5)bisphosphate (PIP_2). By hydrolysis, it generates the second messengers inositol(1,4,5)trisphosphate (IP_3) and diacylglycerol (DAG) which in turn control several distinct cellular processes. Also independent of this enzymatic function, PLCG1 has been found to influence functions such as actin cytoskeleton reorganization, migration and proliferation [494-503]. Some of these functions are exploited in cancers or cancer cells of breast, head and neck or prostate to drive progression and metastasis [504-508].
STATs are usually directly recruited to the activated receptor leading to their phosphorylation, dimerization and nuclear translocation with subsequent induction of target

gene transcription [509]. The phosphorylation of STAT5B has been shown to depend on GAB1 expression: GAB1 overexpression led to increased SHP2 phosphatase activity dephosphorylating its substrate STAT5B [432,510,511]. Besides different physiological activities, STAT5B is involved in several tumorigenic processes, *e.g.* in cancers of the hematopoietic system, prostate or head and neck [509,512-515].

Interestingly, all MACC1 interactors show spatiotemporal and/or functional association with GAB1. The apparent similarities between MACC1 and GAB1 and their possible consequences should be investigated in more detail. This will be discussed later (4.3).

4.2.2 MACC1 rewires cellular signaling networks to exert malignant functions

In view of the previously iterated literature and our findings that MACC1 overexpression facilitates ERK activation in CRC cells, we were interested to further illuminate the MACC1 signaling landscape. For this, we employed the recently developed methods of DigiWest and PamChip® assay. With both methods, we confirmed and extended our knowledge of MACC1 signaling. We found the phosphorylation of several proteins to be upregulated in MACC1 overexpressing CRC cells. These proteins are involved in different crucial signaling pathways indicating a central role for MACC1 in the control of their activity.

We demonstrate the hyperactivation of ERK signaling by MACC1 overexpression indicated by the increased phosphorylation of activating sites on MEK, ERK1/2 and RSK [58]. MACC1 has already been shown by us and others to exert some of its tumorigenic effects via ERK signaling [100,191,208,233,241,243,244,250,267-270]. Here, we revealed a more precise picture including signal strength and duration. Moreover, we demonstrated that MACC1 facilitates ERK signaling in response to HGF and EGF.

MACC1 has been shown to induce β-catenin signaling leading to increased expression of target genes such as MYC, Cyclin D1/E and MMPs [237,250]. The MACC1/AKT/β-catenin axis also confers chemoresistance and stemness characteristics such as sphere formation by activating EMT [219,221,223,225,253]. This study corroborated the induction of β-catenin via MACC1: The inactivating phosphorylation of GSK3β, a negative regulator of β-catenin, at S9 and the activating phosphorylation of β-catenin at S552 were upregulated by MACC1 overexpression. Both phosphorylations have been shown to be conferred by AKT and PKA [75,516-521]. While a MACC1/AKT/β-catenin axis has been established already (see above), AKT activity was not increased in the experimental set-up of this study. More interestingly, several members of cAMP/PKA signaling including PKA (regulatory subunit alpha - pS99 and regulatory subunit beta - pS114) and its downstream effectors CREB (pS133) and VASP (pS157) showed elevated activity upon MACC1 overexpression [522-527]. VASP connects upstream signaling to cytoskeleton dynamics and, therefore, controls processes such as cell

adhesion, polarity and motility [526]. CREB is involved in multiple physiologic and homeostatic functions and *in vivo* knock-out has been shown to be lethal. It can act as transcription factor to drive genes implicated in proliferation, survival and migration. Consequently, overexpression and increased activity haven been found to enhance tumor progression and metastasis [528]. In addition, β-catenin and CREB have recently been found to associate in the promotion of lung cancer [529].

FAK stimulation is usually triggered by integrin clustering, *i.e.* when cells interact with the extracellular matrix. This interaction mediates autophosphorylation of FAK at Y397, a central binding site for SRC. Recruitment of SRC leads to its activation via phosphorylation at Y416. In turn, SRC catalyzes the phosphorylation of Y576/577 and Y925 on FAK resulting in its full activation [530,531]. We found that MACC1 overexpression strongly enhanced the phosphorylation of all these five presented sites indicating a clear activation pattern of this pathway. Now, PI3K can bind to pY397 whereas GRB2 interacts with pY925 of FAK. Together with SRC, these interactions link FAK to a multitude of signaling pathways, such as PI3K and ERK, involved in cell survival, invasion and migration. In consequence, FAK and SRC have also been shown to drive tumor progression and metastasis [530-533]. With these findings, we added several new layers to the MACC1 signaling landscape underlining its strong impact on shaping a tumorigenic and metastatic phenotype (Fig. 36).

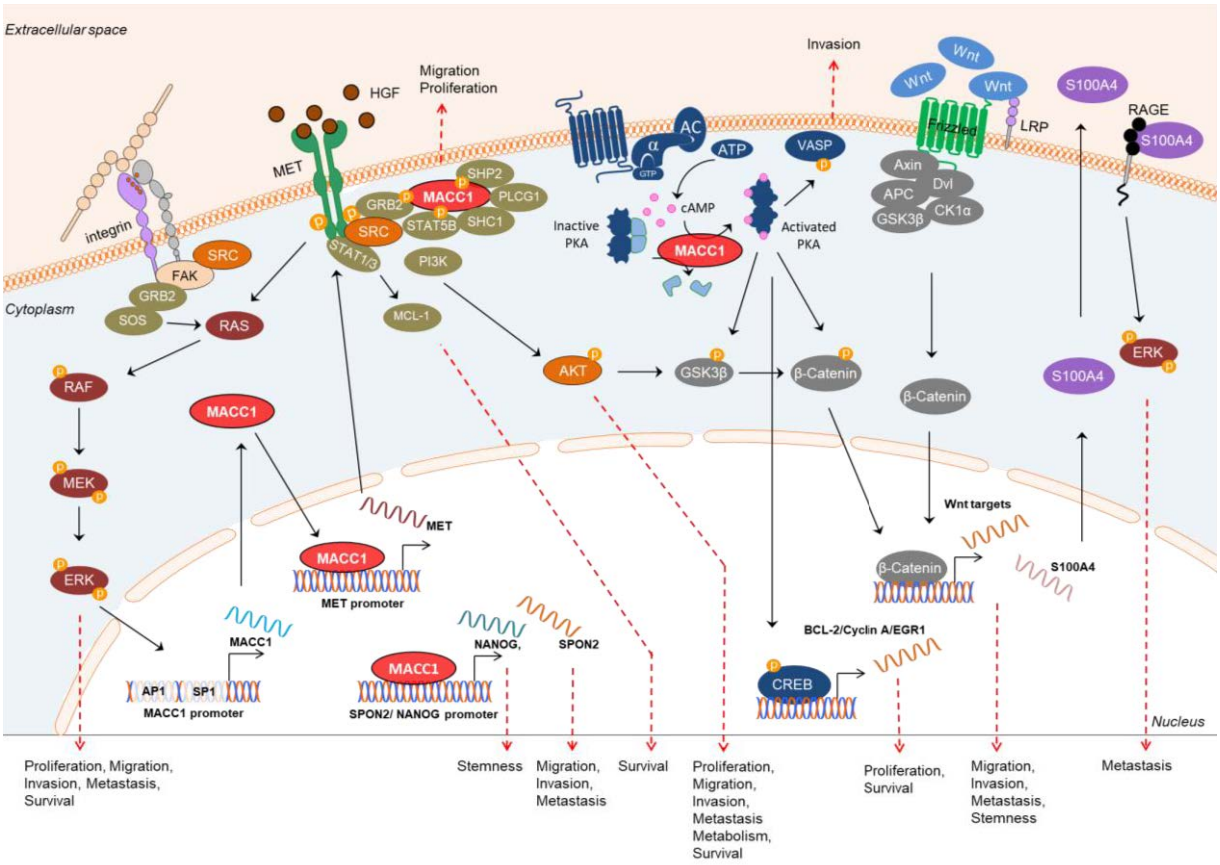


Fig. 36: MACC1 signaling landscape with additional layers. This study revealed several new layers of MACC1 signaling. MACC1 interacts with central signaling components (GRB2, SHP2, SHC1, PLCG1, STAT5B) governing different signaling cascades involved in the control of proliferation, migration and survival. MACC1 was newly found to facilitate FAK/SRC and PKA/CREB/VASP signaling. MACC1 – metastasis associated in colon cancer 1; MET - MET receptor tyrosine kinase; HGF – hepatocyte growth factor; GRB2 - growth factor receptor-bound protein 2; SOS - son of sevenless; RAS - rat sarcoma viral oncogene homolog; RAF - v-Raf murine sarcoma viral oncogene homolog; MEK - MAPK/ERK kinases; ERK - extracellular signal–regulated kinase; AP-1 - activator protein 1; SP1 - specificity protein 1; STAT1/3/5 - signal transducer and activator of transcription 1/3/5; SHC1 - SHC-transforming protein 1; SHP2 - SH2 domain-containing phosphatase 2; PI3K - phosphoinositide 3-kinases; MCL-1 - induced myeloid leukemia cell differentiation protein; NANOG - homeobox protein NANOG; SPON2 - spondin 2; AKT – protein kinase B; GSK3β - glycogen synthase kinase 3 beta; Dvl - dishevelled protein; APC - adenomatous polyposis coli; CK1α - casein kinase I alpha; LRP - lipoprotein receptor-related protein; RAGE - receptor for advanced glycation endproducts; S100A4 - S100 calcium-binding protein A4; FAK - focal adhesion kinase; SRC - non-receptor tyrosine kinase SRC; PKA – protein kinase A; AC - adenylate cyclase; ATP - adenosine triphosphate; GTP - guanosine triphosphate; cAMP - cyclic adenosine monophosphate; VASP - vasodilator-stimulated phosphoprotein; CREB - cAMP response element-binding protein.

Interestingly, most of the target proteins demonstrate similar phosphorylation patterns indicating stringent activation loops. These patterns are typically found in signaling pathways and represent their internal negative feedback loops [534,535]. A very prominent example is the ERK pathway: Activated ERK confers negative phosphorylations to its upstream regulators SOS, RAF and MEK thereby suppressing its own activity [536-539]. As described

above (1.2.2), aberrations in these tightly controlled signaling cascades can have detrimental consequences. Already minor changes can exert a major impact on cellular function: Duration and strength of ERK signaling have been found to strongly determine cell fate by altering the downstream transcription machinery and subsequent expression patterns of target genes [540-542]. Newer findings show that phosphorylation and recruitment events upon receptor stimulation already happen within a timeframe of seconds [543,544]. This further emphasizes the influence differences in signal strength or duration within minutes - as observed by us and others - can impact on cellular fate [545-547]. Another study showed that the closely related ligands FGF-7 and FGF-10 steer cells to distinct functions (proliferation or migration) through individual signaling duration routines [548]. In this regard, it is very interesting to note that signal duration is determined dependent on the recruitment of SH3 domain-binding protein 4 (SH3BP4) to FGFR. SH3BP4 presents an almost 50% sequence homology to MACC1 [100].

Besides self-controlling mechanisms, signaling pathways intertwine to compensate or congregate with each other, leading to a highly dynamic and precisely adjusted network [26,535]. An ideal example for this presents the signal amplifier GAB1. In a basic setting, GAB1 recruitment to the plasma membrane depends on generation of PIP_3 binding sites in the membrane by PI3K and a phosphorylation of GAB1 by ERK on S552 to release the PH domain from its inactive state. Membrane-bound GAB1 harbors interaction sites for PI3K and the GRB2-SOS-RAS complex which increases downstream signaling of both pathways through positive feedback. Upon growth factor binding, GAB1 is predominantly recruited to the RTK promoting RAS-ERK signaling with reduced influence of PI3K activity. Continuously increasing RAS-ERK signaling negatively feeds back to GAB1 balancing the receptor response. The GAB1 amplifying effect has been found to be essential in response to low growth factors signals [64,549-551]. Apparently, GAB1 presents a key player in mediating downstream signaling below different RTKs. As described above, all RTKs generally involve only a certain set of adaptors and mediators, which shares a great overlap among them. The signaling response differs depending on recruitment partners and patterns [48,449,466,535,543,552]. Our finding that ERK signaling was facilitated by MACC1 overexpression also in response to EGF supports this notion. It further suggests that MACC1 can act as malignant signaling mediator below several RTKs. We have preliminary data demonstrating MACC1-dependent upregulation of ERK signaling in response to FGF, PDGF, IGF and VEGF, but more work has to be done to validate these findings.

In consideration of the current literature, MACC1 signaling harbors possible key players: SHP2, SRC and PKA. SHP2 has already been proven to be a central mediator of RTK downstream signaling, governing different cellular responses involved in tumorigenesis and

metastasis [52-54,428-436,467-475]. More interestingly, several studies found that SHP2 exerts its functions via the activation of SRC [436,553-555]. Concomitantly, SRC has been shown to act downstream of several RTK such as MET, EGFR and PDGFR. On the other hand, SRC is also able to directly phosphorylate different RTKs leading to their activation [530,556]. Song and colleagues interestingly demonstrated the interdependency of MET, EGFR and IGFR signaling with SRC as central mediator in HCT116 and HT29 CRC cells [557]. SRC has also been shown to elicit a GAB1 signaling response dependent and independent of MET [558,559]. Moreover, a recent study revealed that SRC induced breast cancer in a PKA-dependent manner *in vivo*. Here, Beristain and colleagues further demonstrated that low PRKAR1A (leading to increased PKA activity)/high SRC expression characterizes basal-like and HER2 breast tumors with poor prognosis [560]. It is worth mentioning that some studies suggest an SRC-STAT5 axis promoting malignant phenotypes [561-563]. In our experimental setting however, we could not observe an elevated STAT5B activation at Y699 with MACC1 overexpression in response to HGF (results not shown), but this might present a mode of action in another cellular context depending on growth factor and entity.

On this basis, we propose the novel MACC1/SHP2/SRC/ERK and MACC1/PKA/SRC/CREB axes conferring a malignant phenotype in response to different growth factor signals. So far, only very little is known about a link of RTKs to PKA signaling [564]. MACC1 might present a new connection of these pathways. In total, our data suggest that MACC1 is able to rewire the finely tuned pathway networks to promote specific routes of signaling, resulting in tumor enhancing and metastasis inducing functions.

4.2.3 Intervention in MACC1 signaling reveals new possible treatment strategies

Finally, to validate our signaling findings on the functional level and reveal potential drug intervention points in MACC1-dependent signaling, we preformed clonogenic assays in the presence of distinct inhibitors. We chose inhibitors of MET (Critzotinib, PHA-665752), SHP2 (GS493), MEK (Cobimetinib) or SRC (Dasatinib, Bosutinib, PP2) to interfere with the newly discovered MACC1 pathways. Interestingly, all inhibitors restricted MACC1-induced colony formation to control cell level.

More than half of the inhibitors used in this study have already been employed in clinical settings and, therefore, present ideal candidates for time and cost efficient drug repurposing in the context of MACC1 signaling [337]: Crizotinib is an ATP mimetic found to inhibit the RTKs MET, ALK and ROS-1. It has been employed in several clinical studies predominantly for the treatment of lung cancer and was firstly FDA approved in 2014 for the treatment of ALK positive advanced or metastatic non-small cell lung cancer. Further clinical

investigations including other entities are ongoing [565-570]. Crizotinib has also been reported to be effective in treatment of CRC *in vitro* and *in vivo* [571-573]. PHA-665752 blocks the phosphorylation of MET at Y1234/1235 and with this its catalytic activity. While it has not been used in clinical trials, PHA-665752 showed cancer inhibiting effects *in vitro* and *in vivo* in different entities such as ovarian cancer, melanoma and CRC [574-578].

GS493 is a very recently emerged, competitive inhibitor of SHP2. The group of Birchmeier and colleagues developed it and demonstrated its efficacy in tumor inhibition for lung, breast and pancreatic cancer *in vitro* and *in vivo* [56,579,580]. Cobimetinib, a MEK1/2 inhibitor, has reported effects in the treatment of CRC and melanoma in different clinical trials usually in combination with other therapeutic agents [581-583]. It received its first FDA approval for the treatment of unresectable or metastatic BRAF V600 mutation-positive melanoma in 2015, but more clinical trials with different indications are in progress [584,585].

Dasatinib, an inhibitor of the kinases SRC and ABL, was approved by the FDA for the treatment of chronic myeloid leukemia in 2006. It has been shown to exert moderately positive effects in clinical trials for entities such as breast and prostate cancer whereas further trials are ongoing [586-588]. Clinical trials for CRC presented unsuccessful, but Dasatinib was effective *in vitro* and *in vivo* [589,590]. Another SRC/ABL inhibitor, Bosutinib, was also approved for CML treatment in 2013. Bosutinib further demonstrated positive therapeutic activity in patients with advanced breast cancer and moderate response in other solid tumors including CRC [591-593]. Further clinical trials are in progress [594]. Bosutinib has also been reported to reduce CRC growth *in vitro* and *in vivo* [595,596]. While PP2 has not been applied to clinical trials yet, Bertotti and colleagues showed that it inhibits anchorage-independent growth of breast cancer cells [436]. It has been further demonstrated to resensitize ovarian cancer cells to chemotherapeutic therapy and restrict tumor growth as well as metastasis formation in a mouse model for CRC metastasis [597,598].

Many clinical studies discussed above involve the combined treatment of different targeted or non-targeted inhibitors. Combinatorial drug treatment represents one of the currently predominant approaches to overcome drug resistance in cancer and achieve better therapy response [599]. In this regard, it is interesting to note that Song and colleagues combined PHA-665752 and Dasatinib to elicit a stronger apoptotic response in CRC cells [557]. Ruess *et al.* used GS493 in combination with the MEK inhibitors selumetinib or trametinib to overcome resistance against MEK inhibition in pancreatic and lung cancer *in vitro* and *in vivo* [56]. These findings demonstrate the potential of combinatorial treatment in MACC1 signaling and should be elucidated further. In this context, it would also be worth to test combinations of MACC1 signaling inhibitors with statins to potentially tackle MACC1 from two different sides.

Further investigations should also address the specificity of respective inhibitors: As described above, some inhibitors display inhibitory effects for several targets. For instance, the SRC inhibitors Dasatinib and Bosutinib also target ABL whereas PP2 has been shown to additionally act against ZAP-70, Janus kinase 2 (JAK2), stem cell factor receptor KIT, EGFR and PKA. Dasatinib demonstrated activity against PDGFR and EGFR as well [600]. Besides MET, Crizotinib also inhibits the RTKs Anaplastic Lymphoma Kinase (ALK) and ROS-1 [565]. The SHP2 inhibitor GS493 further presents off-target effects for PDGFR and SRC [601]. All of these off-target effects have to be taken into account for future research and considerations of therapeutic intervention in MACC1-induced signaling.

Additionally, cell cycle and apoptosis assays could characterize the precise effect inhibitor intervention in MACC1 signaling exerts on the cellular phenotype. Naturally, more functional assays have to be employed to investigate the intervention potential on MACC1-dependent proliferation or migration. After this, drug application *in vivo* could validate our findings and possibly provide additional treatment options for the clinics.

The finding that all tested inhibitors show approximately the same effect additionally suggest a high level of pathway crosstalk or a signaling cascade which is mainly mediated via the MACC1-induced MET overexpression as reported previously [215]. This is contradicted by our findings that MACC1 overexpression led to increased activation of most downstream targets including ERK, β-catenin, CREB and SRC already in unstimulated conditions (0 min). In contrast, MET overexpression has already been reported to activate downstream signaling independent of ligand binding [602]. We are currently working on a MET knock-out model with concomitant MACC1 overexpression which could shed more light on this issue. It would be further illuminating to employ these cells as well as MACC1 knock out cells or inhibitor treated cell to the DigiWest technology. This would reveal changes in pathway activation and, therefore, determine the specific signaling cascade order.

Altogether, our data provide promising intervention points in the MACC1 signaling landscape. Still, some work must be done to entirely define the spatiotemporal regulation of cascades and specific therapy possibilities suitable for the clinical setting.

The findings above are also particularly interesting for additional reasons: For instance, MACC1 protein levels appear to have a crucial influence on cellular signaling given the fact that signaling intervention reverts the overexpression effect on colony formation. This is in line with the original finding that patients with high MACC1 expression have a higher probability for metachronous metastasis and poorer survival prognosis than those with low MACC1 expression. MACC1 overexpression also determined cellular responses such as proliferation, migration and colony formation [100]. Several follow-up studies corroborated

our findings of the prognostic and phenotypical characteristics of MACC1 expression levels for metastasis and patient survival in more than 20 solid tumor entities [215]. This phenomenon has been reported for other proteins as well. For instance, the overexpression of SHP2, GRB2 or SRC have also been shown to influence cellular phenotype and cancer progression associated with poor prognosis [479,480,486,532,603-605]. On the other hand, physiological MACC1 also appears to be involved in exerting malignant functions: Otherwise, specific targeting of MACC1-dependent functions with statins would not have been successful *in vitro* and *in vivo* in this and the previous study [274]. Melvin *et al.* further showed that physiological MACC1 plays a role in embryonal development. MACC1 knock down led to severe craniofacial defects in the developing zebrafish [606]. In this study, we additionally demonstrated that MACC1 knock-out strongly reduced CRC cell proliferation. Some preliminary data suggest that this also remains true for colony formation ability. All results further emphasize the strong malignant influence MACC1 expression confers to cells. Still, some issues remain to be addressed. A valid opportunity should be the generation of certain mouse models to determine the role of physiological and overexpressed MACC1 in development and cancer in an *in vivo* situation. Our group currently works on the creation of conditional (inducible) and ubiquitous knock-in mice models via the Rosa26 locus to reflect physiological and/or varying MACC1 expression levels in several tissues as well as a MACC1 knock-out mouse model [607,608]. This could lead to even more crucial findings by reflecting an *in vivo* situation.

4.3 Conclusion & Outlook

With this study, we demonstrated that the statin family represents potent transcriptional inhibitors of MACC1 in different cancer entities. Their individual potency to reduce MACC1 expression can be attributed to biophysical properties such as hydrophobicity and subsequent biochemical consequences. The additional ability to specifically restrict MACC1-dependent functions *in vitro* as well as tumor progression and metastasis formation *in vivo*, shows the promising potential to repurpose them as therapeutic agents against cancer. While the transcriptional inhibition of MACC1 cannot account for the entire anticancer effect, the disturbance of the mevalonate pathway and subsequent molecular consequences represents a central process. However, there are different possible intersections or mergers of both functional interventions leading to a combined hinderance of cancer progression. Moreover, several studies, including ours (Gohlke & Zincke *et al.*, submitted), document the chemopreventive character of statins with positive results in different, large patient populations.

Because of the usually long latency of a treatment effect and the predominant fatality of cancer diagnosis, the discovery and employment of biomarkers is inevitable for chemoprevention [399]. They help identifying the population at high risk for a certain disease course (prognostic biomarker) or can predict responsiveness to treatment (predictive biomarker) [425]. In view of the current literature and our present data, we recommend chemoprevention with statins in patients stratified for MACC1 expression. This is especially sensible because MACC1 serves as direct target of statins. For the evaluation of the predictive character of MACC1 upon statin treatment, more clinical research is warranted. Here, a combined approach with P53 status might be of interest as Moon and colleagues independently suggested P53 status as prognostic and predictive marker for statin therapy [366].

To eventually translate our findings to the clinics, known side effects of statins, such as muscular syndromes (*e.g.*, myalgia, myositis or rhabdomyolysis), would have to be assessed on a case-by-case basis. The lack of validated diagnostic tools or clinical criteria besides creatinine levels may impede a precise evaluation of statin side effects [609]. Otherwise, side effects could be alleviated by using statins in combination with other drugs to achieve additive or synergistic effects with concomitant reduction in dose and side effects. Several studies already support this advantageous use of statins in combination therapy [341,610,611]. This is especially interesting because we revealed new inhibitors of MACC1-dependent signaling which could be tested in combination with statins to potentially elicit a more comprehensive MACC1 inhibition.

In this regard, we identified and confirmed pY-dependent interactions of MACC1 with crucial signaling proteins: SHP2, GRB2, SHC1, PLCG1 and STAT5B. Based on our current results, SHP2 and GRB2 bind to the phosphorylated Y379 peptide whereas SHC1 and STAT5B interacted with pY789 and pY365, respectively. PLCG1 binding was rather unspecific: It showed the strongest interaction with pY379 followed by pY789 and contradicting results for Y365. In consideration of the domain and binding motif architecture, the interaction between SH2 domain and the respective pY-site on MACC1 represents the most probable route of protein engagement but several other binding options could be assumed in the highly flexible and dynamic signaling process. Apparently, MACC1 recruits the interactors to govern a sophisticated signaling network in response to different RTKs, including MET and EGFR, exerting a malignant phenotype. SDM of Y379 and Y789 restricted MACC1-induced signaling as well as cellular functions such as proliferation and migration. Moreover, targeted intervention in the newly proposed MACC1/SHP2/SRC/ERK and MACC1/PKA/SRC/CREB axes with inhibitors of MET, MEK, SHP2 and SRC blocked MACC1-dependent colony formation. In total, our data suggest that MACC1 is able to rewire the finely tuned pathway

networks to promote specific routes of signaling, resulting in tumor enhancing and metastasis inducing functions.

MACC1 obviously displays several similarities with the multi-adaptor and signaling mediator GAB1 (compare 4.2.1 and 4.2.2). Preliminary data on this matter suggest that MACC1 is able to uphold its signaling promoting functions also under GAB1 knock-down. We are currently working on a GAB1 knock-out cell model to elucidate this in more detail. The structural and possible functional similarities to SH3BP4 should also be researched in more detail. Especially, the recent findings of our group that MACC1 is also involved in endocytosis (Imbastari *et al.*, submitted) indicate an additional role of MACC1 in endosomal signaling, which is known to regulate central cellular processes [612-614].

As described above, all RTKs generally involve only a certain set of adaptors and mediators eliciting a variety of pathways with distinct functions. The signaling response differs depending on recruitment partners and patterns [48,449,466,535,543,552]. Considering this, it would be of utmost importance to uncover the spatiotemporal regulation of MACC1 interactor recruitment and downstream signaling cascades. We are currently working on a MET -/- model to clarify the dependency of MACC1 signaling on receptor mediated activation and adaptor recruitment. Furthermore, we are trying to elucidate the spatiotemporal regulation of MACC1 signaling with size exclusion chromatography, 2D-Blue Native/SDS-PAGE and total internal reflection fluorescence microscopy (TIRFM) [615-620]. These findings would significantly improve the knowledge about MACC1 signaling with concomitant increase in intervention precision and quality.

In addition, the inhibitor studies should be extended to further cell models, cellular functions and inhibitor combinations. This includes the combination of different targeted inhibitors as well as the combination of targeted inhibitors with statins. It might be of additional interest to include inhibitors of GRB2 or PKA since they have been found effective in reducing tumor progression *in vitro* and *in vivo* [605,621-626]. Both apparently play a crucial role in MACC1 signaling and GRB2 has additionally been demonstrated to control basal activity of FGFR and connected SHP2 activation [627-629]. An additionally promising strategy presents the development of an antibody blocking the MACC1 interaction sites (Y379 and Y789) similar to monoclonal antibody therapy already employed in cancer therapy [630].

Finally, another approach should be the generation of certain mouse models to determine the role of physiological and overexpressed MACC1 in development and cancer in an *in vivo* situation. Our group currently works on the creation of conditional (inducible) and ubiquitous knock-in mice models via the Rosa26 locus to reflect physiological and/or varying MACC1 expression levels in several tissues as well as a MACC1 knock-out mouse model [607,608].

Signaling of inhibitor treated vs untreated mice should be assessed via WB, DigiWest and/or MS to draw a highly precise picture of the MACC1 signaling network *in vivo*.

The potential of MACC1 as multimodal biomarker and therapeutic target has not entirely been exploited yet. It could serve as crucial target in future cancer therapy and, particularly, the promising and advancing field of cancer prevention.

5. Abbreviations

5-FU	5-fluoruracil
18q	q-arm of chromosome 18
ABL	Abelson murine leukemia viral oncogene homolog 1
AC	adenylate cyclase
AKT	protein kinase B
ALK	Anaplastic Lymphoma Kinase
ANOVA	analysis of variance
AP-1	activator protein 1
APC	adenomatous polyposis coli
AREG	amphiregulin
ATP	adenosine triphosphate
ATPase	adenosinetriphosphatase
AUC	area under the curve
BAD	Bcl-2-associated death promoter (BAD) protein
BAX	Bcl-2-associated X protein
BCL2	B-cell lymphoma 2
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BSA	bovine serum albumin
C2	C2 domain
cAMP	cyclic adenosine monophosphate
CBL	casitas B-lineage lymphoma proto-oncogene
CDC4	cell division control protein 4
cDNA	complementary DNA
CDK4/6	cyclin-dependent kinase 4/6
CH1	CH1 domain
CHK1/2	checkpoint kinase 1/2
CIMP	CpG island methylator phenotype
CK1α/δ	casein kinase 1 alpha/delta
CKI	cyclin-dependent kinase inhibitor
CMS	consensus molecular subtypes
CMV	cytomegalovirus
CpG islands	cytosine-guanosine dinucleotides
CRC	colorectal cancer
CREB	cAMP response element-binding protein

CSC	cancer stem cells
CSF1R	colony stimulating factor 1 receptor
CTC	circulating tumor cells
CXCL12	C-X-C motif chemokine 12
CYP	cytochrome P450 enzyme
DAG	diacylglycerol
DKFZ	German Cancer Research Center
dMMR	MMR deficiency
DMSO	dimethylsulfoxide
Dox	doxycycline
Dvl	dishevelled protein
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMAP-II	Endothelial-Monocyte-Activating Polypeptide–II
EMBL	European Molecular Biology Laboratory
EMT	epithelial-to-mesenchymal transition
ER	estrogen receptor
EREG	epiregulin
ERK	extracellular signal–regulated kinase
FAK	focal adhesion kinase
FAP	familial adenomatous polyposis
FASL	FAS ligand
FDA	U. S. Food and Drug Administration
FGFR	fibroblast growth factor receptor
FLT-3	FMS-like tyrosine kinase 3
FPP	farnesyl-diphosphate
G6PD	glucose-6-phosphat-dehydrogenase
GAB1	GRB2-associated binder 1
GBM	glioblastoma multiforme
GEF	guanine nucleotide exchange factor
GFs	growth factors
GGPP	geranyl-geranyl-diphosphate
GLI	glioma-associated oncogene
GPCR	G protein-coupled receptor
GRB2	growth-factor-receptor-bound protein 2
GSK3β	glycogen synthase kinase 3 beta

GTP	guanosine triphosphate
HCC	hepatocellular carcinoma
HDL	high-density lipoprotein
HER2	human epidermal growth factor receptor 2
HGFR	hepatocyte growth factor receptor
HGF/SF	hepatocyte growth factor/scatter factor
HIF1	hypoxia inducible factor 1
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
HNPCC	hereditary nonpolyposis colorectal cancer
HP1	heterochromatin protein-1
HRP	horseradish peroxidase
HR	hazard ratio
HTS	high-throughput drug screening
IGF2R	insulin-like growth factor 2 receptor
IL6R	interleukin 6 receptor
IP_3	inositol(1,4,5)trisphosphate
IRS	insulin receptor substrate
ITGB8	integrin beta 8
JAK2	Janus kinase 2
KIT	stem cell factor receptor
KRAS	Kirsten rat sarcoma viral oncogene homolog
LDL	low-density lipoprotein
lncRNA	long non-coding RNA
LPA	Lysophosphatidic acid
LRP	lipoprotein receptor-related protein
MACC1	metastasis-associated in colon cancer 1
MCL-1	induced myeloid leukemia cell differentiation protein
MEK	MAPK/ERK kinases
miRNA	microRNA
MKP	MAPK phosphatases
MLH1	MutL homolog 1
MMP	matrix metalloprotease
MMR	mismatch repair
MOI	multiplicity of infection
mRNA	messenger RNA
MSH2	MutS protein homolog 2

MSI	microsatellite instability
MS	mass spectrometry
MSS	microsatellite stable
mTORC1	mammalian target of rapamycin complex 1
MYH	MutY homolog
NANOG	homeobox protein NANOG
NF-1	Neurofibromin 1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
OATP1B1	organic anion-transporting polypeptide
OCT4	octamer-binding transcription factor 4
OR	odds ratio
p14ARF	ARF tumor suppressor
PD1	programmed cell death protein 1
PDGFR	platelet-derived growth factor receptor
PDK1	3-phosphoinositide-dependent protein kinase-1
PD-L1	programmed death-ligand 1
PGF	placental growth factor
PH	Pleckstrin homology domain
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKA	protein kinase A
PLCG	phospholipase C gamma
PP2A	protein phosphatase 2
PRD	proline rich domain
PTEN	phosphatase and tensin homolog
PTP	protein tyrosine phosphatase domain
PTP-SL	receptor-type tyrosine-protein phosphatase R
pY	phosphorylated tyrosine
qRT-PCR	quantitative real-time polymerase chain reaction
RAS:GTP	active/GTP-bound form of small GTPase RAS
RAGE	receptor for advanced glycation endproducts
Rb	retinoblastoma protein
RET	rearranged during transfection receptor tyrosine kinase
RNAi	RNA interference
ROS	reactive oxygen species

RPL32	ribosomal protein L32
RR	risk ratio
RSK	90 kDa ribosomal S6 kinases
RTK	receptor tyrosine kinase
RWE	real-world evidence
S100A4	S100 calcium-binding protein A4
SDM	site-directed mutagenesis
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SH2/3	Src homology 2/3
SH3BP4	SH3 domain-binding protein 4
SHC1	SH2 domain containing transforming protein
SHP2	SH2 domain-containing protein tyrosine phosphatase 2
shRNA	small hairpin RNA
SIC1	cyclin-dependent protein kinase-1
siRNA	small interfering RNA
SLUG	zinc finger protein SNAI2
SMAD4	SMA & MAD homolog 4
SNAIL	zinc finger protein SNAI1
SOS	son of sevenless
SP1	specificity protein 1
SPON2	spondin 2
SRC	non-receptor tyrosine kinase SRC
STAT	signal transducer and activator of transcription
TCF4	Transcription factor 4
TCF/LEF	T-cell factor/lymphoid enhancer-binding factor
TGFBR2	transforming growth factor beta receptor 2
TNFR	tumor necrosis factor receptor
TP53	tumor suppressor P53
TRAIL	tumor necrosis factor related apoptosis inducing ligand
TSC1/2	tuberous sclerosis 1/2
TSS	transcription start site
TUM	Technical University Munich
TWIST1	twist basic helix-loop-helix transcription factor 1
UVB	ultraviolet B
VASP	vasodilator-stimulated phosphoprotein

VEGFR	vascular endothelial growth factor receptor
VHL	von Hippel–Lindau tumor suppressor
WB	Western blot
Wnt	wingless/integrated
ZAP-70	ζ-chain (T-cell receptor)-associated protein kinase 70 kDa
ZEB	zinc finger E-box binding homeobox 1
ZFP36	zinc finger protein 36 homolog

6. Statement of Contribution

Ich versichere hiermit, dass die von mir vorgelegte Dissertation eigenständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt wurde. Ich versichere, dass alle aus anderen Quellen übernommenen Daten und Konzepte, sowie Ergebnisse aus Kooperationsprojekten unter Angabe der Referenz gekennzeichnet sind.

Außerdem versichere ich, dass mir die aktuelle Promotionsordnung bekannt ist und ich mich nicht anderwärts um einen Doktorgrad bewerbe, bzw. noch keinen entsprechenden Doktorgrad besitze. Diese Arbeit wurde in gleicher oder ähnlicher Form nicht einer anderen Prüfungsbehörde vorgelegt.

Berlin, den 06.01.2020 _____

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